Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 80 AUGUST-SEPTEMBER, 1952 No. 4

Tocopherol Administration to Patients with Dupuytren's Contracture; Effect on Plasma Tocopherol Levels and Degree of Contracture.* (19692)

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Investigations of the effect of tocopherol administration on Dupuytren's contracture were initiated by Steinberg(1), who reported the results of treatment in 7 patients, who were given 300 mg of vit. E by mouth daily for periods ranging from one to 8 weeks. In 6 out of the 7 patients the contracture disappeared. More recent studies by Steinberg(2) on a larger group of patients have, on the whole, confirmed his original findings. Favorable results with this treatment have also been reported by Scott and Scardino(3) and by Thomson(4). In the group of patients studied by Thomson 10 hands were classified as early cases and 8 as late or chronic cases. Of the 10 early cases 8 had a definite extension defect, but in no instance were the fingers contracted closer than 90° to the palm. In these patients full extension of the fingers was restored following treatment with 200 mg of tocopherol daily for 12 to 20 weeks. The 8 chronic cases exhibited marked thickening of the palmar aponeurosis with fixation of the affected fingers in the palms; in these patients the tocopherol administration resulted in a softening of the palmar masses. On the whole, however, the improvement in the latter group was not very marked but nevertheless in 4 patients sufficient to make the hands suitable for surgical treatment.

In contrast to these rather favorable results are observations by Langston and Badre(5) and by King(6) who treated respectively 5 and 13 cases of Dupuytren's contracture with similar doses of tocopherol, but in no instance noted any benefit.

Experimental. The present investigation included 19 patients with Dupuytren's contracture, 14 men and 5 women, with a total of 26 contracted hands. The mean age of the men at the onset of the study was 74 years, and of the women 77 years. The lesion was bilateral in 6 of the men and one of the women. The right hand was affected in 16 cases (12 men, 4 women), the left hand in 10 cases (8 men, 2 women). In 5 of the 26 hands one or more of the fingers were contracted closer to the palm than 90°. In the remaining 21 hands the lesion could be characterized as moderately severe or slight. In none of the male patients was the coexistence of Peyronie's disease found. Treatment with 300 mg of dl-alpha-tocopherol acetate (Ephynal Acetate) daily was given to all the patients for a period of 300 days, and it was ascertained through supervision by a nurse that the patients swallowed the administered capsules. After discontinuation of treatment the patients were followed for another 350 days. Plasma tocopherol was determined before the onset of

* Vitamin studies in middle-aged and old individuals. IX.

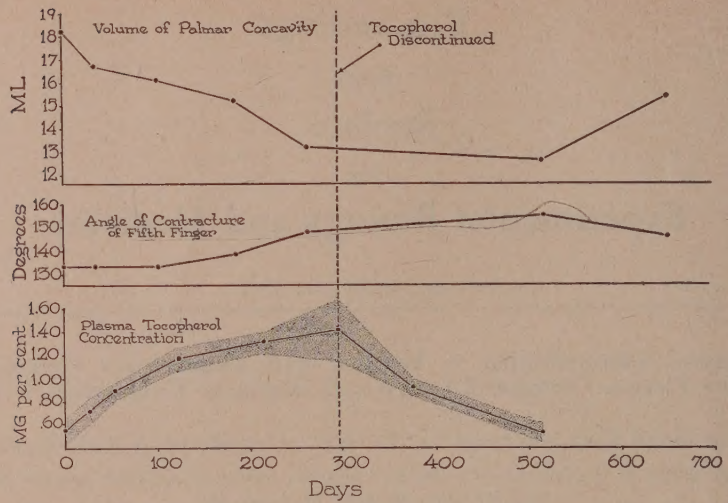

FIG. 1. Effect of alpha-tocopherol acetate administration (300 mg daily) and subsequent discontinuation on plasma tocopherol level and on degree of hand lesion in 19 old individuals with Dupuytren's contracture. The shaded area represents one s.d.

the treatment and at various intervals during and after tocopherol administration by the method of Quaife and Biehler(7). The procedure used for registering the degree of contracture was to make plaster casts of the hands under standard conditions. During the application of the plaster the patient's upper arm rested on a table with the elbow joint held in 90° flexion and the wrist in maximal dorsal flexion. A plaster bandage consisting of 3 layers of quick setting plaster gauze was then applied from a point proximal to the wrist to the finger tips. During this application, until the plaster had stiffened, a firm and constant pressure was exerted against the volar aspect of the distal part of the fingers, at the same time as the plaster gauze by a nurse was carefully molded against the surface of the palm and the individual fingers. These permanent casts obtained during maximal passive dorsal flexion of the wrist and fingers formed the basis for the evaluation of changes in the degree of contracture resulting from the tocopherol administration. For quantitative registration of the changes in the appearance of the hands 2 measurements were chosen, which give a fairly good impression of 2 of the main characteristics of the contracture, namely the degree of concavity of the palm and the angle formed between the fifth finger and the direction of the palm. Fine sand was applied to the palmar aspect of the plaster casts and the surface of the sand adjusted as accurately as possible to the plane formed by the thenar and hypothenar prominences. By subsequent weighing of the sand an estimate of the volume was obtained. This volume in normal individuals varies between 10 and 18 ml depending on the size of the hand. Tracings were made on paper directly from the casts and the angle formed between the volar surface of the 2 proximal phalanges of the finger and the general direction of the distal part of the volar aspect of the palm itself measured by a protractor. This measurement was possible in only 23 of the 26 hands. The angle in normal individuals usually varies between 190° and 205°.

Results. The effect of alpha-tocopherol administration on the plasma tocopherol concentration and on the hand measurements described above is illustrated in Fig. 1. The figure shows that the mean plasma tocopherol concentration before the onset of treatment was 0.55 mg %, a value which is significantly lower than the mean value of 1.02 mg % previously reported by the present authors(8) for this age group. Following the alpha-tocopherol administration the plasma level rose gradually and slowly to reach, after 300

days, a mean value of 1.37 mg %, or a concentration two and a half times as high as the original. After discontinuation of the alpha-tocopherol intake the plasma tocopherol concentration again decreased. As seen from the figure the mean value after 75 days had fallen to 0.88 mg % and, when measured after 215 days, had reached practically the same level as observed before the treatment was started, namely 0.53 mg %.

In no instance of moderately severe contracture did the treatment result in disappearance of the extension defect of the fingers. However, as seen in Fig. 1 a definite, though moderate improvement was noted during the prolonged alpha-tocopherol administration. In 23 of the 26 hands the palmar concavity became less marked. The determinations of the mean volume of the concavity in the 26 hands showed a decrease from 18.2 to 13.2 ml during the 300 days of alpha-tocopherol administration, a change which is statistically significant. In the first 215 days following discontinuation of the treatment a further slight improvement took place (mean volume 12.6 ml). On examination of the hands 250 days after discontinuation of therapy the amelioration had again regressed somewhat (mean volume 15.3 ml).

The angulation between the fifth finger and the direction of the distal part of the palm showed definite improvement in 15 of the 23 hands. In 7 of the remaining 8 hands the changes were only slight although with a tendency to an increase of the angle, whereas in one hand the angle remained constant. In none of the hands did the contracture of the fifth finger, when comparison was made between the first casts and the casts obtained at the end of the tocopherol treatment, became worse. A change in the mean value of the angle was not noticeable until after about 200 days of alpha-tocopherol administration, at which time the value had increased from 133.0° to 138.4°. This change is statistically significant. After 300 days of treatment the value had increased further to 147.7°, and this improvement continued during the first 215 days after discontinuation of the alpha-tocopherol therapy, at which time the value of 154.0° was reached, corresponding to a total mean increase in the angle of 21°. In the subsequent 135 days the mean value for the contracture angle of the fifth finger again decreased to 146.3°.

No untoward symptoms were noted during the prolonged alpha-tocopherol administration. A few of the patients volunteered the information that they could move their fingers and hands a little better; on the whole, however, the moderate objective improvement was not registered by the senile patients, who also before the onset of the treatment seemed unconcerned about the condition of their hands.

Discussion. In view of the fact that the lesion in the present group of patients on the whole was less severe than in the cases studied by Thomson, the smaller and slower response to alpha-tocopherol administration observed in our group becomes notable. In spite of this difference in the degree of improvement observed, the results of the present investigation would, nevertheless, support the contention of Steinberg and of Thomson that the administration of alpha-tocopherol in large daily doses has an effect on the lesion in Dupuytren's contracture.

It is possible that the smaller effect observed in the present group of patients may be due to the advanced age of the patients. Since the contracture in our cases probably had existed for a far longer period than in the patients treated by Thomson it seems further likely that the less marked effect noted by us on the degree of contracture of the fifth finger may have been conditioned by the existence of degenerative arthritic changes in the finger joints. Such changes are known to occur in contractures of long standing, and, if present, would tend to obscure an eventual therapeutic effect on the connective tissue of the palmar aponeurosis.

Summary. 1. Treatment with 300 mg of alpha-tocopherol acetate daily was given to 19 old patients with Dupuytren's contracture (26 contracted hands) for a period of 300 days. Following discontinuation of the treatment the patients were followed for another 350 days. Before therapy and at various intervals during and after alpha-tocopherol administration determinations were made of the

plasma tocopherol concentration and permanent plaster casts prepared of the affected hands in maximal passive dorsal flexion. 2. The alpha-tocopherol administration resulted in a slow but steady rise of the mean plasma tocopherol level from 0.55 to 1.37 mg %; after discontinuation of the treatment the concentration again fell to the original level. A moderate, but statistically certain effect of the therapy was observed on the condition of the hands. Thus, during the treatment the volume of the palmar concavity decreased from 18.2 to 13.2 ml, and the angle between the fifth finger and the palmar surface increased by 14.7°. The improvement continued for some time after discontinuation of the therapy, but some regression had taken place on examination 350 days after the end of the alpha-tocopherol treatment.

Funds and material for this study were provided through the generosity of Hoffmann-La Roche, Inc.

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Received May 6, 1952. P.S.E.B.M., 1952, v80.

Effects of Potassium on the Guinea Pig after Barbiturate Administration. (19693)

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Lamson and coworkers(1) have demonstrated that glucose and other organic substances can modify the anesthesia produced by barbiturates in various animal species. The well-known relation of potassium to glucose in the movements of the latter in and out of cells, and the implication of this cation in carbohydrate metabolism and neuromuscular processes(2) suggested a trial of potassium under conditions similar to those described for glucose. Preliminary work indicated that potassium could reinduce deep levels of anesthesia in guinea pigs recovering from hexobarbital anesthesia. The results of the investigation which followed are the subject of this report.

Methods. Guinea pigs were used throughout the study. Hexobarbital sodium† was used as the anesthetic agent, fresh solutions being made up for each experiment. Potassium chloride (KCl) and sodium chloride (NaCl) were administered as isotonic solutions (1% and 0.85% respectively). All doses of anesthetic or inorganic salt were injected intraperitoneally.

Results. The first experiments were attempts to reinduce deep anesthesia in guinea

TABLE I. Effect of KCl on Guinea Pigs Recovering from Barbiturate Anesthesia.

Guinea pig	Hexobarbital, mg/kg	Duration of anesthesia, min	KCl, mg/kg	Additional anesthesia, min
1	60	32	100	7
2	60	35	100	6
3	60	31	200	8
4	60	33	200	7
5	40	13	20	2
6	40	9	25	3½
7	40	14	50	5
8	40	15	50	5
9	40	26	400	13
10	40	20	400	10

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† The author is greatly indebted to Dr. John C. Seed of Sterling-Winthrop Research Institute for a generous supply of this compound.

TABLE II. Effect of NaCl on Guinea Pigs Recovering from Barbiturate Anesthesia.

Guinea pig	Hexobarbital, mg/kg	Duration of anesthesia, min	NaCl, mg/kg	Additional anesthesia, min
1	60	34	170	
2	60	33	170	
3	40	24	50	
4	40	24	200	0
5	40	10	200	
6	40	19	400	
7	40	11	400	

pigs recovering from the narcosis produced by 40 to 60 mg of hexobarbital per kg. KCl was injected when the pig showed spontaneous activity or would squeal and make definite running movements if picked up by the skin over the back. In 10 experiments in 10 pigs, the injection of KCl was followed by an apparent return to deeper anesthetic levels (limpness, failure to move when placed on side or back) in all cases. Table I shows the time required to recover to the point described above following hexobarbital and then KCl. Depicted in Table II is the failure to affect the course of recovery with injections of NaCl in 7 animals.

The next step was to investigate the effects of potassium on the unanesthetized guinea pig. Forty-two experiments were performed in 13 guinea pigs. KCl produced apparent excitement, with scurrying, twitching, and nodding movements,‡ followed by a disturbance of the righting reflex lasting about 30 to 120 seconds, and a suggestion of slight muscular weakness, for the same period of time, following the larger doses. Some animals required 25, others as much as 200 mg per kg, to produce these effects. In all instances, the pigs were capable of standing and moving about if placed on their feet. In addition, 6 of the animals were given 800 mg per kg, with similar results, except that after 20 to 45 minutes, having apparently recovered from the acute effects of the injected potassium, the animals showed respiratory difficulties, weakness, occasionally convulsive movements, and death. Respirations were noted to cease before cardiac standstill, as described by Amberg and Helmholz(3). Except for the situation immediately before death, there was never observed any relaxation of the type seen in the barbiturate-KCl treated animals described above.

‡ These symptoms were also observed (prior to the onset of anesthesia) after KCl in the first group of experiments described.

In the next group of experiments, attempts were made to obtain deep anesthesia by combining large doses of KCl with doses of hexobarbital which either had no effect on the pigs or produced only a disturbance of the righting reflex. Eight experiments were performed in 8 animals. Doses of 20 mg per kg of hexobarbital were injected, followed in 4-5 minutes by doses of 100 to 400 mg of KCl. In 7 out of the 8 experiments, the period of "excitement" was immediately followed by a state indistinguishable from moderately deep stages of anesthesia (flaccidity, inability to rise when placed on their backs or sides, or to stand when put on their feet). A number of animals exhibited roving uncoordinated movements of their eyeballs, and inability to respond to pinprick. The data, with duration of effects noted, are shown in Table III. In no case was there seen quite the degree of narcosis or duration of effect that could be obtained with large doses of hexobarbital.

The last group of experiments comprised a study of the ability of hexobarbital to alter the response to potassium when the drugs were employed together in doses that were fractions of the amounts required to produce effects when either drug was administered alone. The method utilized was the following: First, a group of 13 pigs was injected intraperitoneally with successively increasing doses of hexobarbital, starting with 10 mg per kg and doubling the dose until an effect was produced. In all cases, at least 24 hr elapsed between administration of individual doses. As a measure of effect, abolition of the righting reflex was chosen. All untreated pigs invariably righted themselves when placed on their backs, so that abolition of this reflex was a reliable indication of definite effect. Also, this relatively clear-cut endpoint eliminated the subjective element in the evaluation of response. The results were as follows: no pig showed any effect from 10 mg per kg, 3 pigs showed disturbance of righting reflex from 20 mg per kg,

TABLE III. Effects Produced by KCl in Guinea Pigs Given Non-Anesthetic Doses of Barbiturate.

Guinea pig	Hexobarbital, mg/kg	KCl, mg/kg	Duration of anesthesia, min	Effects noted: Loss of righting reflex	Flaccidity	Loss of response to pin prick	Roving pupils
1	20	100	6	+	+		
2	20	200	19	+	+	+	+
3	20	200	6	+	+		
4	20	400	12	+	+		+
5	20	400	15	+	+	+	+
6	20	400	13	+	+		
7	20	400	15	+	+	+	+
8	20	400	3	+	Slight		

and 10 pigs required 40 mg per kg. In most instances this latter dose produced rather deep anesthesia.

This same procedure was then followed for KCl, starting with 25 mg per kg. One pig showed disturbance of the righting reflex with 25 mg per kg (and not with 15), 5 with 50 mg per kg, 4 with 100 mg per kg, and 3 required 200 mg per kg to show an effect.

The last step was to use the barbiturate and inorganic salt together. For this study, each pig was given a dose of hexobarbital which was 1/4 of the smallest dose required to produce a disturbance of the righting reflex. After 4-5 minutes (the maximal time required for definite evidence of onset of anesthesia when effective doses of hexobarbital were used) the pig was injected with a fraction of the minimal dose of KCl needed to produce an effect when injected alone. With this technic, abolition of the righting reflex was obtained with the following fractions of the "minimal effective dose" of KCl: 1/2 in 2 pigs, 1/4 in 4 pigs, 1/5 in 2 pigs, 1/10 in 4 pigs, and 1/40 in 1 pig. These results confirm the other studies in demonstrating an unmistakable modification of the response to KCl by the previous administration of hexobarbital. This alteration of response can thus be shown both with anesthetic doses of barbiturate and with doses of barbiturate which have no demonstrable effect.

Discussion. Although this study was initiated by theoretical considerations of the glucose effect of Lamson *et al.*(1), the results described in this paper are in no way offered as an explanation of this effect. The widespread participation of potassium in such diverse processes as nerve impulse formation, acetylcholine formation, carbohydrate metabolism, electrolyte transfer, etc.(2,4) makes extremely difficult the interpretation of such simple experiments as those described here. The report of Stewart(5) on the ability of barbiturate anesthesia to increase markedly the blood concentrations of potassium achieved in the rabbit after intraperitoneal injection of KCl is of considerable interest in regard to the work being reported. The results obtained in the studies on the righting reflex could be explained by a similar effect in the guinea pig. However, the relegation of hexobarbital to the role of a mere elevator of the blood potassium concentrations achieved after injection of potassium salts leaves some observations unexplained, such as the ability of potassium to reinduce, in a pig recovering from anesthesia, or to induce, in a pig given ineffective doses of anesthesia, a state indistinguishable from that obtained with anesthetic doses of barbiturate. The essential absence of signs of anesthesia in pigs given sublethal or even lethal doses of potassium suggests qualitative differences which require the postulation of a more complex joint action of barbiturate and potassium.

Summary. A study of the effect of hexobarbital on the response of the guinea pig to injected potassium has revealed the following: 1) Potassium, even in maximally tolerated doses, is incapable of producing anesthesia when injected alone. 2) Potassium is capable of inducing a state indistinguishable from deep narcosis in pigs recovering from hexobarbital anesthesia. 3) Potassium can produce moderately deep narcosis in guinea pigs

given subanesthetic doses of hexobarbital. 4) Following the injection of small doses of hexobarbital, potassium can abolish the righting reflex in a fraction of the dose required when injected alone.

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Received May 15, 1952. P.S.E.B.M., 1952, v80.

Effect of Cortisone on Lipids of Serum, Liver and Testes in Intact and Adrenalectomized Rats.*† (19694)

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Cortisone therapy in man increased the serum concentration of cholesterol and phospholipid, and decreased serum neutral fat (1,2). The increase of serum cholesterol, total and esterified, has been reported in rats treated with cortisone(3). In rabbits, adrenal extract and cortisone administration also increased cholesterol, lipid phosphorus and total fatty acids of serum(4-6). ACTH was shown to cause fatty infiltration of the liver in the rabbit(6,7) and rat(8). Adrenalectomy prevented fat mobilization into the liver in mice given ACTH(9). Cortisone caused no fatty infiltration of the liver in intact or adrenalectomized mice(10). However, lipid granules were found in hepatic cells of intact rabbits treated with cortisone(6). Cortisone was not found to cause a severe involution of testis in rats(3,11). ACTH was found to cause a slight reduction in the weight of rat testes with atrophy of the Leydig cells(12).

This study reports the effect of cortisone administration on total cholesterol and lipid phosphorus concentration of serum, liver and testes in adrenalectomized rats maintained with desoxycorticosterone pellets, as compared with the effects of the hormone in intact rats.

Experimental. The animals used in this study were 56 adult male rats of the Sprague-Dawley strain, selected for uniformity and with a weight range of 270 to 320 g. They received Rockland Rat diet and tap water *ad libitum*. Vit. A (200 I.U.) and vit. D (100 I.U.) were given orally once a week.

The experimental groups are shown in Table I. Adrenalectomy was performed through a flank approach. Each rat was maintained with a 15 mg pellet of desoxycorticosterone acetate (DCA).§ For the first 2 days following operation, the animals received by mouth 5% glucose in 0.3% saline, after which they were kept on the same diet as the other rats. Cortisone acetate¶ was given subcutaneously, 4 mg every day (0.5 ml in 0.9% saline) for 28 days. The rats were weighed twice weekly. The cortisone-treated rats lost about 50 g. At different times, daily food intake was weighed in each group. Blood was drawn under ether

* Research of the endocrine division is supported in part by Grants-in-Aid from the American Cancer Society, and by a research grant from the National Institutes of Health.

† The author is indebted to Drs. Lawson Wilkins, Walter Fleischmann, Lytt Gardner, Gordon Kennedy and Samuel Silverman for helpful suggestions. Thanks are due to Mr. Henry Schulte, Mrs. Mary Ellen Crafton and Miss Betty Youngblood for technical assistance.

‡ Fellow of the André and Bella Meyer Foundation (France) on recommendation of the Institute of International Education, New York.

§ The author is indebted to Dr. F. E. Houghton, of Ciba Pharmaceutical Products, Summit, N. J., for Percorten pellets.

¶ The author is thankful to Dr. L. V. Curtin of Merck and Co., Rahway, N. J., for cortisone.

TABLE I. Liver Weight and Concentration in Total Cholesterol and Lipid Phosphorus. (Mean value ± stand. dev. of group.)

	Exp. groups	Liver wt, g: Total	Liver wt, g: Per 100 g B.W.	Total cholesterol, mg: Total	Total cholesterol, mg: Per g of liver	Lipid P, mg: Total	Lipid P, mg: Per g of liver
I	Normal (10)*	10.99 ± .30	3.45 ± .78	34.29 ± 1.4	3.14 ± .28	7.82 ± .55	.714 ± .037
	Normal + cortisone (12)	9.758± .67	4.30 ± .29	30.4 ± 2.7	3.10 ± .29	7.43 ± .72	.764 ± .074
II	Normal (6)	10.55 ±1.04	2.96 ± .37	32.98 ± 5.3	3.12 ± .17	7.66 ±1.20	.725 ± .06
	Normal + cortisone (6)	10.60 ± .97	4.15 ± .95	33.10 ± 1.06	3.12 ± .46	8.06 ± .88	.759 ± .08
	Adrenal × + DCA (10)	11.58 ±1.00	3.16 ± .22	33.75 ± 2.32	2.92 ± .26	11.50 ± .40	.996 ± .06
	Adrenal × + DCA + cortisone (12)	9.25 ± .73	3.55 ± .35	27.82 ± 2.16	3.03 ± .34	6.19 ± .81	.670 ± .07

* No. of animals.

anesthesia from the abdominal aorta into centrifuge tubes as soon as possible after the animal was anesthetized, since a rise in blood cholesterol may occur after 5 to 7 minutes of anesthesia(13). The blood was immediately centrifuged and the serum separated. The testes were quickly removed, and weighed to the nearest 5 mg; the tunica albuginea was stripped from each testis before weighing in an effort to eliminate contributions of tissue other than the tubule-interstitial cell complex. Each testis was then put in a metal cup and desiccated immediately. The livers were carefully dissected and weighed to the nearest 5 mg, divided into several weighed pieces of approximately 1 g each, and desiccated immediately in metal cups.

Total cholesterol and lipid phosphorus determination in serum. Total cholesterol was determined by the method of Bloor, Pulkan and Allen(14), using a Klett-Summerson photoelectric colorimeter. Lipid phosphorus was determined by the method of Youngburg and Youngburg(15) as modified by Hawk, Oser, and Summerson(16), using an Evelyn photoelectric colorimeter.

Total cholesterol and lipid phosphorus determination in testes and liver. The dry tissue was ground with C.P. sea sand in a mortar and then transferred to a 50 ml centrifuge tube. The mortar, pestle and cup which contained the tissue specimen were washed down with 25 ml of ethanol-ether mixture and the washings were collected in the centrifuge tube. The tube was attached to a vertical Graham coiled condenser for refluxing, and the contents boiled for 40 minutes. Tube was then raised out of the water bath and the condenser washed with 5 ml of ethanol-ether mixture. When the tube was cooled, it was centrifuged for a few minutes and the supernatants filtered using fat-free filters. The tissue was extracted a second and third time in the same way, the extracts combined with the initial filtrate and made to 100 ml in a volumetric flask with ethanol-ether mixture. Duplicate

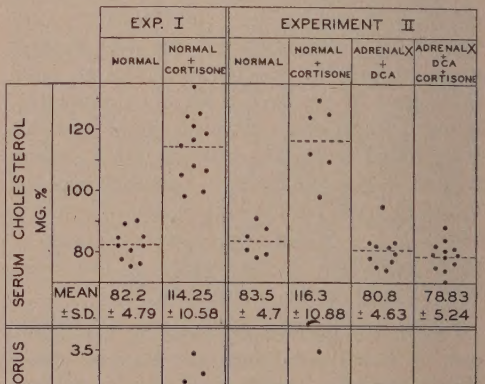

FIG. 1. Total cholesterol and lipid phosphorus in serum of the rats in different experimental groups. Dotted lines represent mean value in each group.

TABLE II. Testes Weight and Concentration in Total Cholesterol and Lipid Phosphorus. (Mean value ± stand. dev. of group.)

	Exp. groups	Testes wt, g: Total	Testes wt, g: Per 100 g B.W.	Total cholesterol, mg: Total	Total cholesterol, mg: Per g of testis	Lipid P, mg: Total	Lipid P, mg: Per g of testis
I	Normal	3.196±.118	.937±.164	5.861±.316	1.832±.090	1.158±.16	.363±.063
	Normal + cortisone	2.802±.254	1.231±.126	4.754±.505	1.702±.183	.887±.18	.317±.050
II	Normal	3.343±.157	.938±.089	6.180±.480	1.848±.112	1.069±.15	.319±.047
	Normal + cortisone	2.946±.241	1.146±.097	5.231±.630	1.775±.130	.985±.11	.338±.052
	Adrenal × + DCA	3.279±.222	.894±.075	5.696±.363	1.650±.16	1.087±.172	.332±.043
	Adrenal × + DCA + cortisone	2.974±.257	1.130±.175	5.120±.396	1.725±.144	.805±.133	.270±.041

TABLE III. Level of Significance of the Salient Differences in Results as Tested by "Students" T Test (20).

	Differences tested		t	D.F.*	P.†
Serum cholesterol	Normal against normal + cortisone	Exp. I	8.32	20	<.001
	" " "	" II	6.17	15	<.001
Serum lipid P.	" " "	" I	6.11	20	<.001
	" " "	" II	3.11	10	<.02
	Normal against adrenal × + DCA	" II	1.38	16	>.1 <.2
Liver lipid P.	" " "	" II	5.60	14	<.001
	Normal against adrenal × + DCA + cortisone	" II	3.05	16	<.01

* D.F. = degrees of freedom. † P = probability of the difference being due to chance.

aliquots of this stock solution were used for total cholesterol and lipid phosphorus determinations as described for serum.

Results. 1) The results of total cholesterol and lipid phosphorus analyses of the sera are shown in Fig. 1. Cortisone therapy was associated with an increased total cholesterol in intact rats, but did not increase that of adrenalectomized rats maintained with DCA. Cortisone administration increased serum lipid phosphorus of intact rats but had no effect on the adrenalectomized rats maintained with DCA. A slight decrease of serum lipid phosphorus was noted in adrenalectomized rats maintained with DCA, compared with the values for intact rats. This change was not significant (Table III).

2) *Effect of cortisone therapy on the liver.* The results are shown in Table I. Cortisone administration did not modify liver weight or total cholesterol concentration in either intact or adrenalectomized rats. Cortisone therapy did not change lipid phosphorus concentration in the liver of intact rats. The adrenalectomized rats maintained with DCA had a significant increase in liver lipid phosphorus, compared with intact rats. Cortisone treatment slightly decreased liver lipid phosphorus in adrenalectomized rats, compared with intact rats. This decrease was not significant (Table III).

3) *Effect of cortisone therapy on testes.* Cortisone therapy decreased the weight of the testes, but the weight per 100 g of body weight was normal or slightly increased over control values (Table II). Total cholesterol and lipid phosphorus content of the testes decreased on cortisone therapy in both intact and adrenalectomized rats, but the changes were not significant.

Discussion. The administration of cortisone did not increase total cholesterol and lipid phosphorus of serum in adrenalectomized rats maintained with DCA, although these were increased in intact animals. Other workers had noted that administration of ascorbic acid

(10-100 mg intramuscularly) increased serum cholesterol of the intact rat but had no effect on the adrenalectomized rat(17). Our findings cannot be explained by a difference in food intake, as the intact rats and adrenalectomized rats with DCA, both showed a decrease in food intake during cortisone therapy (18). Since serum sodium and potassium levels were within normal limits in every group, the results cannot be explained by a change in the dilution of plasma.

The treatment of adrenalectomized dogs with DCA lowered their blood phospholipids (19). In our experience, the serum lipid phosphorus of adrenalectomized rats maintained with DCA was slightly but not significantly lower than normal.

Although the cholesterol concentration of the liver was not changed in any group, the liver lipid phosphorus concentration was increased in the adrenalectomized rats maintained with DCA. This finding is difficult to interpret.

Summary. 1. Cortisone therapy increased serum cholesterol and lipid phosphorus of intact rats but did not have this effect in adrenalectomized rats maintained with DCA. 2. Adrenalectomized rats maintained with DCA showed a significant increase in liver lipid phosphorus. This was restored to normal by a dosage of cortisone which was without effect on the liver lipid phosphorus of the intact rat. 3. Cortisone therapy decreased testicular weight of intact and adrenalectomized rats, but caused no change in the concentrations of cholesterol and lipid phosphorus of the testes.

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Received May 26, 1952. P.S.E.B.M., 1952, v80.

Oncolytic Effect of Viruses in Tissue Cultures.* (19695)

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When Levaditi and Nicolau(1) described the propagation of herpes virus in mouse epitheliomas they were seeking a substrate on which to propagate the virus. These authors and others(2-4) later demonstrated that when avian pest virus localized and propagated in mouse tumor tissue, it exerted therein a cytolytic effect. This initiated similar studies with other viral agents such as neurotropic

* This study was supported by funds provided under Contract AF 41-607-42 with the USAF School of Aviation Medicine, Randolph Field, Texas.

vaccinia(5), rabies(6), vaccinia(7), and lymphogranuloma venereum(8), but these resulted at most in some vitiating effect on transplants of the parasitized tumors. On the other hand, Virus III seemed to enhance the malignancy of a transplantable tumor of rabbits(9). Recent *in vivo* studies have indicated that Russian Spring-Summer encephalitis (RSSE)(10,11), St. Louis encephalitis (SLE) (12), and Bunyamwera virus(13) would parasitize and destroy transplantable mouse sarcomas. An avian lymphoid tumor propagated *in vivo* was destroyed by RSSE, SLE, Japanese B and West Nile viruses(14). Egypt 101 virus destroyed human epidermoid carcinoma growing in x-irradiated rats and in tissue culture(15). It is obvious that one of the major hazards to the application of virus therapy for human cancer may be the pathogenic effect of some viruses on the patient. Many of the agents thus far found to be lethal to the tumors are unfortunately no less lethal to the host.

The oncolytic effect of viruses on experimental tumors of rodents can be demonstrated *in vitro*. The roller tube culture technic described by Cameron(16) involves the propagation of plasma-embedded tumor implants with ox serum ultrafiltrate† as the principal nutrient in the supernate. Two transplantable sarcomas have been studied: 1, a methylcholanthrene-induced anaplastic sarcoma of the rat;‡ and 2, a chemically-induced fibrosarcoma in the C_3H mouse. Both killed their hosts at approximately 2 months after inoculation. Four surgically excised tumors of human origin were also studied for their response to viral infection.

Methods. The tumors were minced with scissors and 4 to 5 pieces (about 1.0 mm in diameter) were implanted in coagulating chicken plasma, to which ox serum ultrafiltrate (30%), Hanks solution with phenol red indicator (60%) and chick embryo extract (10%) were added in 1.0 cc quantity. One hundred units each of penicillin and of streptomycin were added to each tube. The nutrient fluids were replaced twice per week and otherwise were adjusted to optimal pH with 1.4% $NaHCO_3$ when needed. Thirty to 40 tubes were assembled with each tumor and to groups of 5 a different virus was added, so diluted that the 0.05 ml inoculum contained either 1,000 LD_{50} or 1,000 hemagglutinating units of virus. The viruses were inoculated with the fresh implant or after the viable tumor had grown well (48-72 hours). All of the implants were examined under low power magnification during the next 7-14 days. Each experiment was repeated at least 3 times. The viruses used and their menstruums were as follows: Newcastle disease (NDV) (California strain), mumps (Enders strain), and influenza A in chick embryo chorio-allantoic fluid (CAF); and rabies (CVS strain), herpes simplex (MF strain), neurotropic influenza A, and St. Louis encephalitis (SLE) (Hubbard strain) in mouse CNS tissues. Lansing poliomyelitis virus (LCR) was used in cotton rat CNS. All of the virus preparations were clarified at 5,000 rpm for 20 minutes and were stored in sealed ampules in the CO_2 chest.

Results. The rat sarcoma implants rapidly metabolized the nutrient fluid causing the indicator to turn yellow within 24 hours. The untreated cells grew into the periphery as a solid mass of round cells. Herpes, influenza A (CAF) and LCR viruses exerted little effect on its growth properties. Rabies virus seemed to enhance the growth of this tumor tissue whereas SLE virus destroyed the implant and its outgrowth within 3 days (Fig. 1). The nutrient fluid for the SLE-treated tissues remained basic. When tumors were infected with SLE virus at implantation they grew poorly from the start and cytolysis was complete by the second and third days. When SLE virus was added 2 days after implantation it killed the tumor tissue within 48 hours. Neurotropic influenza A virus caused much deterioration of the implant and its outgrowth but only after a delay of 5-6 days. The cytolytic effect of the latter virus was not complete.

The fibro-sarcoma implants from C_3H mice demonstrated fibroblastic outgrowths within 24-36 hours. By 48 hours each implant was surrounded by a grossly visible "halo" of

† Microbiological Associates, Bethesda, Md.

‡ Strain initiated by the M. D. Anderson Foundation for Cancer Research, Houston.

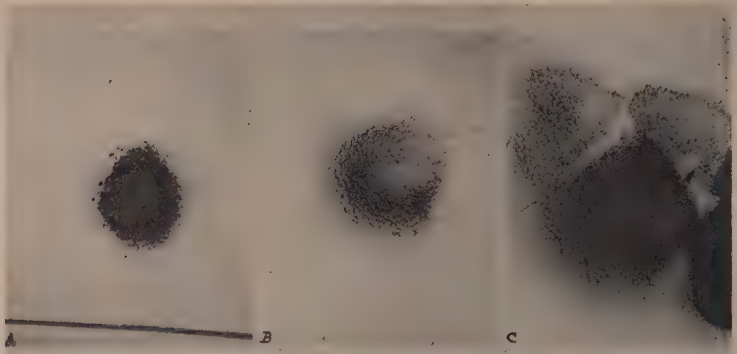

FIG. 1 RAT SARCOMA (ANDERSON)—48 HOURS

A +SLE Virus B No Virus C Rabies Virus

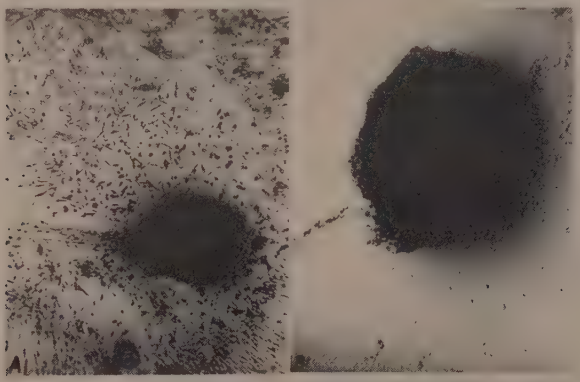

FIG. 2 MOUSE SARCOMA

A Normal B Effect of SLE Virus—48 hours after inoculation

HUMAN MALIGNANT PAROTID TUMOR (Clon)

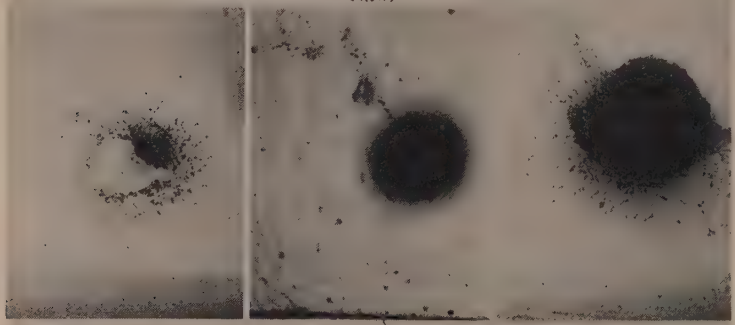

FIG. 3

10 days after adding NDV Virus | 8 days after adding SLE Virus | 10 days after adding Rabies Virus

cellular growth. Plasma liquification appeared in some areas of outgrowth causing the elongated cells to round up. Such tumor outgrowth often equalled the size of the implant within 72 hours. When NDV, rabies, influenza A (CAF), and LCR viruses were added to 48-hour-old implants there was no apparent change in the growth pattern. The rabies-infected tissues appeared to grow slightly more extensively. SLE virus caused fragmentation of the "halo" within 24 hours and death of all tissues by 48 hours (Fig. 2). Twenty-four hours after adding neurotropic influenza virus, the cellular outgrowths appeared granular and some degeneration of cells was evident at 48 hours. Most of the tissues infected by the latter virus were degenerated at 72 hours; however, this latter effect was not complete: the cells were shrunken, opaque and degenerating, but occasional healthy cells were observed throughout the following week.

Of the human tumors studied, a melanoma of low malignancy, which was excised from the leg of a 39-year-old white female, did not grow in tissue cultures. A scirrhous fibrosarcoma from the jaw of a 54-year-old white male also grew poorly in tissue cultures.

An undifferentiated parotid tumor§ from a 44-year-old white male grew well and implants were inoculated with virus on the fourth day. Those tissues to which NDV and rabies viruses were added continued to grow very well. SLE virus caused a visible shrinkage of the growth "halo" within 24 hours, which became granular and degenerated within 48 hours. By the sixth day the entire implant was disintegrated (Fig. 3). Neurotropic influenza A virus caused a similar sequence which appeared to be completely destructive by the eighth day.

A human anaplastic adenocarcinoma¶ within the abdominal cavity was collected with ascitic fluid. Implants thereof grew extensively over the wall of the tube and acidified the nutrient fluid within 24 hours. When virus was added after 24 hours of growth, NDV did not interfere with growth, rabies caused it to grow more extensively and SLE caused the cells to become pyknotic, crenated and many cells were disintegrated. On the fifth day following the addition of virus, the implants with rabies virus were growing profusely, while those with SLE were acellular.

Discussion. The tissue culture technic is a useful method for determining the virus susceptibility of a growing tumor. It is obvious that not all viruses exert the same cytopathogenic effect. There are some (NDV, LCR, influenza) without apparent influence, whereas another (rabies) may actually speed up the rate of growth of tumor tissue. This lack of effect has already been studied *in vivo* with influenza and herpes(17). The destruction of tissue was also reflected by the color of the indicator in the nutrient fluid. Actively growing tissues quickly acidified the medium, whereas such fluids remained basic when the tissues were killed. This color change has been found useful in studying the effect of poliomyelitis virus in tissue cultures(18).

Neurotropic influenza virus eventually destroyed the mouse fibro-sarcoma and interfered with growth of the rat sarcoma; however, we can postulate that the intact animal would have been immunized to this virus before the oncolytic process was complete. The oncolytic effect of neurotropic influenza virus contrasted sharply with the innocuous effect of chick embryo adapted virus of the same type. Perhaps a street strain of rabies virus might reverse the cell stimulating effect of the fixed rabies virus described herein. SLE virus appeared to kill the tumor implants within 48 hours after inoculation and would appear to be the agent of choice for all of the tumors (rodent and human) studied. Its pathogenic properties in the human, however, might cause one to feel that we would only be substituting one serious affliction with another if this virus was to be inoculated into a cancer patient.

If the antibody producing mechanism could be suppressed, perhaps a less lethal agent such as neurotropic influenza A virus might eventually destroy the tumor. Influenza is a rather common disease and it might be expected that the patient is already refractory to infection with such an agent, thereby obviating its effectiveness from the start. By incorporating the patient's serum into the nutrient fluid, the

§ Supplied by Dr. Truman Blocker, U. Texas Med. Branch, Galveston, Texas.

¶ Supplied by Dr. Paul Howe, M. D. Anderson Hospital, Houston, Texas.

immune status of the host may be determined simultaneously with the oncolytic effect of the virus.

The authors subscribe to the contention of Toolan and Moore(15) that a preliminary determination of viral sensitivity for each tumor should precede any consideration of therapy through viral infection. The tissue culture technic described provides a relatively rapid method of study by which a wide range of viruses can be studied for their effect on each human tumor. By this method, too, new viruses can be surveyed for possible cytopathogenic effect, with the hope that an agent with selective effect on the tumor can be either found or developed.

Summary. Tumor implants, growing in roller tube cultures, demonstrated a marked oncolytic response to St. Louis encephalitis infection. Two human and 2 rodent tumors were destroyed completely by SLE virus, less completely by neurotropic influenza virus. Infections with Lansing poliomyelitis, Newcastle disease, herpes and chick embryo adapted influenza A viruses did not interfere with growth of tissue implants. Rabies virus appeared to stimulate the growth of such tumor implants. The tissue culture technic can be used as a survey tool in rapidly determining the cytopathogenic effect of viral agents on tumors.

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Received May 27, 1952. P.S.E.B.M., 1952, v80.

Effect of Antibiotics on Obstructive Appendicitis in Rabbits.* (19696)

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Previous work from this laboratory has shown that the cecal appendage of man, the chimpanzee and the rabbit possess a secretory capacity(1,2). When the orifice of the appendix is obstructed the "closed-loop syndrome" accompanied by perforation occurs. In animals in which a secretory capacity cannot be demonstrated, obstruction of the cecal appendage does not lead to perforation(3,4). Recently it has been shown that antibiotics reduce the mortality from appendicitis and its complications in man(5,6).

It seemed desirable to test the effects of antibiotics on the obstructed appendix in the rabbit to ascertain: 1) whether antibiotics reduce the mortality in this animal, and if so 2) whether they exert their effects through reduction in secretion or by other mechanisms.

Experimental procedure. Adult white rabbits of both sexes, weighing 1.8 to 4.3 kg were anesthetized with nembutal-ether and under surgical asepsis the appendicocecal juncture

* This research received support from the following funds: Augustus L. Searle Fund; Austen B. Cargill Fund; G. Nelson Dayton Fund; Graduate School of the University of Minnesota.

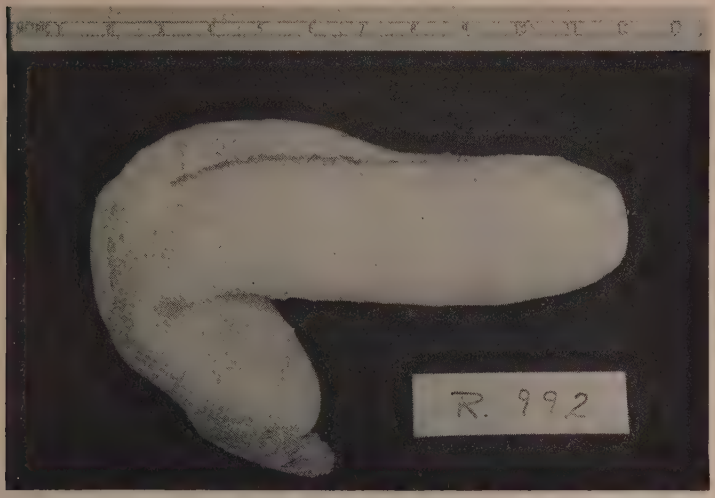

FIG. 1. Parenteral penicillin for 30 days. Large distended appendix resembling a mucocele. No perforation could be found.

was occluded by a silk ligature passed between the gut wall and the blood vessels. The vascular supply to the appendix was undisturbed. The appendical lumen was not emptied. Trauma to the appendix was avoided. A full diet of water and commercial rabbit pellets was offered to all animals immediately after surgery. Control animals received no treatment. Parenteral antibiotics were administered as penicillin in oil 60,000 units twice a day or aqueous streptomycin 0.25 g twice a day in the back muscles beginning 8-12 hours after surgery. Local antibiotics were given as a single dose of aqueous penicillin 50,000 units or aqueous streptomycin 0.5 g each in 2.5 cc of solution deposited in the appendical lumen with a small needle introduced through the cecum. To determine the duration of therapy necessary to insure complete recovery, parenteral antibiotics were discontinued after varying time intervals. If the rabbit survived an arbitrary time of 60 days after cessation of parenteral antibiotics or after instillation of local antibiotics it was considered cured and sacrificed for pathological study. In a separate group of 18 animals the actual volume of fluid secreted by the obstructed rabbit's appendix during parenteral antibiotic therapy was collected intra-abdominally in a thin rubber condom connected to the appendical lumen by a small glass cannula.

Results. Perforation of the appendix with peritonitis or abscess formation was proved at autopsy in all control animals and in 95% of those treated with antibiotics. No perforations occurred at the site of ligature, the majority being in the proximal third. The appendix was usually enlarged 2 to 5 times normal size and resembled a mucocele (Fig. 1). Scattered deposits of firm white material frequently occurred throughout the abdominal cavity. They appeared as well encapsulated masses or as grape-like clusters resembling a pseudomyxoma peritonei. However, microscopic examination showed the appendical contents to be more cellular than mucoid and the scattered masses proved to be abscesses containing 5-50 cc of thick white caseous material. In 3 animals despite marked distention of the appendix, no perforation nor evidence of peritonitis could be found. All ligatures were intact and each lumen completely occluded.

Control. All untreated control animals died within 7 days with appendical rupture and peritonitis.

Penicillin. In contrast to the control animals a comparable group treated with parenteral penicillin all survived the 7-day period and were sacrificed for study. The appendix

TABLE I. Survival after Appendical Ligation.

	No. started	No. alive at end of treatment	No. alive 60 days after treatment
Daily parenteral penicillin			
10 days of treatment	6	6	1
14 " " "	8	6*	1
30 " " "	14	6*	6
Daily parenteral streptomycin			
30 days of treatment	11	6*	4
Local penicillin	6		4
" streptomycin	6		6

* Beyond 10 days of treatment some animals died even though receiving parenteral penicillin or streptomycin at the time.

had ruptured in each. Beyond 10 days of treatment some rabbits died while still receiving treatment (Table I). Ten such animals died from the 11th to the 25th day of therapy. All animals that were maintained on penicillin for 30 days after ligation survived the subsequent 60-day period and appeared to be in good health. With shorter periods of treatment the survival rate was greatly reduced. Of 6 animals treated with local penicillin 2 died in 23 days and 4 lived for at least 60 days.

Streptomycin. Of 6 animals treated with parenteral streptomycin for 30 days, only 4 survived the subsequent 60-day period. Five additional animals died between the 3rd and 15th day of therapy. All animals treated with local streptomycin survived the 60-day period.

Secretion. There was no consistent variation to suggest that the amount secreted during parenteral penicillin therapy (75 to 260 cc) or parenteral streptomycin therapy (90-250 cc) was significantly different from the amount secreted by the control group without therapy (85-270 cc). The volume secreted by individual animals varied considerably and showed no correlation with body weight (Table II). It is of special note that appendical perforation did not occur in these animals.

Summary. The high mortality from perforation and peritonitis which uniformly followed untreated obstruction of the rabbit's appendix was definitely reduced by the parenteral or local administration of penicillin or streptomycin. However, appendical perforation was not prevented nor was there a consistent decrease in the secretion of that organ.

TABLE II. Individual Body Weight and Volume Secreted after Appendical Ligation in 18 Rabbits.

	Control		Parenteral penicillin		Parenteral streptomycin	
Days after obstruction	Wt, kg	Vol, cc	Wt, kg	Vol, cc	Wt, kg	Vol, cc
4	2.7	85	2.5	75	2.7	90
4	2.7	270	2.7	260	2.5	245
5	2.5	245	2.5	250	2.7	190
6	4.1	160	4.3	180	4.1	155
11	2.7	155	2.72	155	2.9	250
12	2.7	205	2.72	190	2.7	215

Therefore the beneficial results of these antibiotics must have been due to other mechanisms, in all likelihood the result of their antibacterial activity. Although the mortality was effectively reduced, some animals died while receiving therapy, an indication that antibiotics are no panacea in the treatment of appendicitis and its complications. The importance of secretory pressure as the principal factor in perforation of the obstructed appendix is emphasized by the fact that such perforation was prevented by allowing the pressure to be dissipated into the reservoir of a distensible balloon.

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Received June 10, 1952. P.S.E.B.M., 1952, v80.

Eosinopenia and Degenerating Eosinophilic Leukocytes in Blood.* (19697)

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A mechanism has been described(1) for the eosinopenia that follows the administration of adrenal hormones or the application of stress. The possibility that eosinophiles undergo destruction in the fluid compartments of the body is supported by the finding that different forms of degenerating eosinophilic leukocytes can be observed in the peripheral blood and peritoneal and pleural fluids of the rat under normal and experimental conditions(1,2). Similar observations have been made with human blood *in vitro*(3,4); likewise the destructive influence of adrenal hormones upon eosinophiles has been noted within ligated blood vessels of the dog(4). The time course of the changes in numbers of eosinophiles in the peripheral blood of the rat treated with epinephrine is presented in more detail in this report.

Materials and methods. Six normal female rats (175-200 g) of a modified Long-Evans strain were injected subcutaneously with 1 ml of a freshly prepared 1:5000 solution of epinephrine (Parke-Davis adrenalin chloride) in 1% saline. Tail blood was obtained under light ether anesthesia immediately before and at various times following the injection and diluted with the phloxine-methylene blue glycol stain described by Randolph(5). Since relatively few eosinophiles are detected normally in blood and fewer yet during the eosinopenic state, it was found necessary to count the cells in a larger volume of blood. Standard white cell pipettes were used; blood was drawn to the 1.0 mark and, after dilution, was delivered into both sides of 2 Levy counting chambers and the cells within the entire ruled areas were counted. The lowest number of eosinophiles actually observed at the height of eosinopenia was 32, with as many as 400 cells being present in the early stages of the experiment. As reported previously(1), the degenerating eosinophilic leukocytes are smaller than the normal eosinophiles and are visualized easily in the counting chamber (120 × magnification). Both normal and degenerate eosinophiles were enumerated in each blood sample.

Results and conclusions. Degenerating eosinophilic elements, which we refer to as "small eosinophiles," are observed normally in rat peripheral blood, but in very small numbers (mean ± standard error: 2.2 ± 0.76). The small eosinophilic forms arise from normal eosinophilic leukocytes through degenerative processes involving both the nucleus and

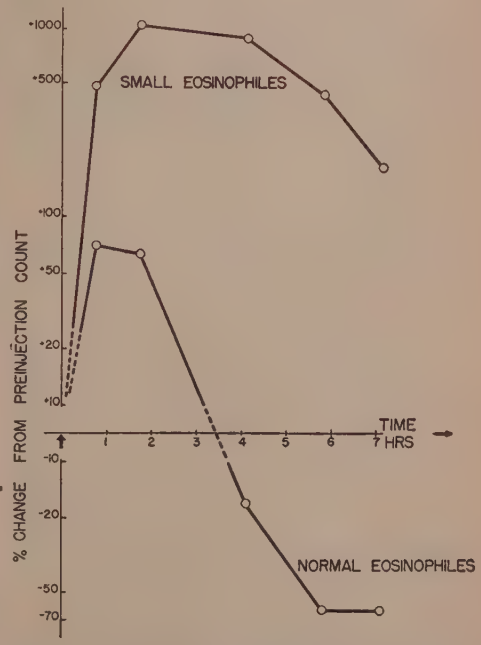

FIG. 1. Variation in the numbers of normal and of degenerating eosinophiles following epinephrine injection. The ordinate is plotted on a logarithmic scale. Note that the numbers of small eosinophiles remain elevated even during the phase of eosinopenia.

* Work done under a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

cytoplasm. The doughnut-shaped nucleus of the normal eosinophile breaks down into fragments that no longer stain with Giemsa and the cytoplasmic granules become densely packed to form masses that stain intensely with eosin or phloxine. Shrinkage of the cell may accompany these reactions. These changes are seen especially well in peritoneal fluid and have been described in more detail elsewhere(1). Following the injection of epinephrine, there is an immediate increase in the numbers of these degenerating elements and fragments in peripheral blood (after 45 min: 11.2 $\pm$ 0.70; $P < 0.01$), and this rise is maintained throughout the duration of the peripheral eosinopenic state (at 4½ hr: 22 $\pm$ 9.2; $P < 0.05$). The data are given in Fig. 1, where the percentage changes of normal and degenerate eosinophiles in peripheral blood are graphed with respect to time after injection.

Careful study of the counting chamber samples suggests strongly that the small eosinophiles arise from the fragmentation of the degenerating eosinophilic leukocyte.

This work lends further support to our contention that eosinophilic cell destruction is a generalized reaction occurring throughout most of the body and that it constitutes an integral part of the mechanism leading to the eosinopenia caused by adrenal hormones and by stress. The present experiments correlate well with our studies on peritoneal and pleural fluids(1,2). Preliminary experiments with cortisone acetate have yielded results similar to those reported here with epinephrine.

Summary. Small numbers of degenerating eosinophilic leukocyte fragments are encountered normally in peripheral blood as well as in other body fluids of the rat. Following epinephrine or cortisone, there is a marked increase in the numbers of eosinophilic leukocyte fragments in peripheral blood, and this increase is maintained throughout the duration of the developing eosinopenia.

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Received June 24, 1952. P.S.E.B.M., 1952, v80.

Effect of Tyrothricin Upon Fibrinolysis Mediated by Streptokinase. (19698)

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The proteolytic dissolution of a fibrin clot, mediated by streptokinase, has been shown by Neter(1) to be inhibited by tyrothricin. These observations were made using whole human plasma and whole cultures of streptococci or crude filtrates. The lytic action, however, has since been shown to be the resultant of 2 simultaneously occurring reactions: 1) Streptokinase + Plasminogen → plasmin; 2) Plasmin + Fibrin → lysis(2). In view of the present clinical use of the fibrinolytic activity of streptokinase it appeared to be of interest to determine, using a more defined system, which of these reactions is inhibited by tyrothricin.

Materials and methods. 1) *Streptokinase.* Partially purified streptokinase preparations derived from culture filtrates of a fibrinolytic strain of *Streptococcus hemolyticus** were used in these studies. 2) *Plasminogen.* Fraction III, obtained from human plasma by the Harvard method of fractionation(3), was prepared by Dr. W. Baumgarten, of these laboratories. 3) Armour's Bovine *Fibrinogen* and Parke Davis Bovine *Thrombin* were used to form the standard fibrin clot. 4) *Streptokinase assay* was performed by the method of

* *Streptococcus hemolyticus* H46A was obtained through the courtesy of Dr. R. L. Christensen of the New York University Medical School.

TABLE I. Inhibition of Streptokinase Activity by Tyrothricin.

Streptokinase preparation	Tyrothricin addition, μg	Time, min	Streptokinase assay
55A	50	0	320 units
	50	30	60
	50	60	27
	Alcohol	60	280

TABLE II. Effect of Tyrothricin-Treated Streptokinase on Plasmin Formation.

Exp.	Units plasmin: Control streptokinase*	Units plasmin: Tyrothricin-treated streptokinase†
1	100	56
2	136	54
3	112	75

* 100 units streptokinase were incubated with 400 units plasminogen.

† 100 units streptokinase incubated with 100 μg tyrothricin before addition of 400 units plasminogen.

Christensen(4), and *plasmin* was determined by a modification of Christensen's plasminogen assay(4). One unit of plasmin is that amount in 0.5 ml of sample that will cause the lysis of a standard fibrin clot in 30 minutes at 35°C. 5) *Tyrothricin.* Wallerstein's tyrothricin was dissolved in alcohol.

Results. Effect of tyrothricin on streptokinase activity. When tyrothricin was incubated with streptokinase for 30 minutes at 35°C, the amount of recoverable streptokinase dropped sharply (Table I). The test was repeated with several preparations of streptokinase, and the degree of inhibition observed varied from 60 to 90%. Although a complete study of the time of inactivation was not made, it was found that almost maximal inactivation occurred within the first 30 minutes of incubation.

Since the tyrothricin was placed into solution in alcohol, the possible inhibitory effect of the added alcohol on this system was determined by incubating the streptokinase with alcohol alone. The data show that, although there was a slight decrease in streptokinase unitage with alcohol alone, the inhibition caused in the same time period by the alcoholic tyrothricin solution was 90% greater.

Tyrothricin-treated streptokinase and plasmin formation. Streptokinase incubated with plasminogen will induce maximal plasmin formation within a 5-minute period(5). However, 100 units streptokinase pre-incubated with 100 μg tyrothricin for 30 minutes were able to activate only 40-70% of the plasmin activated by the normal control streptokinase (Table II).

Tyrothricin-treated plasminogen and plasmin formation. When plasminogen was incubated with tyrothricin for 30 minutes before activation with streptokinase there was no inhibition of plasmin formation (Table III).

Tyrothricin and plasmin activity. Plasmin was formed by the incubation of streptokinase with plasminogen for 5 minutes. The addition of tyrothricin to an aliquot of the preformed plasmin 30 minutes before the determination of the plasmin activity did not induce a significant loss in plasmin activity (Table IV).

Discussion. The pre-incubation of streptokinase with tyrothricin before reaction with plasminogen induced a decrease in streptokinase activity. This was seen in both the streptokinase recovery assays and in the formation of plasmin with the treated streptokinase. However, when tyrothricin was mixed with plasminogen first, there was no effect on the streptokinase activation of plasmin. This suggests that the plasminogen and tyrothricin combine in such a manner as to prevent the streptokinase inhibition by the antibiotic. When preformed plasmin was placed in contact with tyrothricin normal lytic activity oc-

TABLE III. Plasmin Formation from Tyrothricin-Treated Plasminogen.

Tyrothricin	Units plasmin: Exp. 1	Exp. 2
None*	126	144
100 μg†	128	156

* 400 units plasminogen activated by 100 units streptokinase.

† 400 units plasminogen incubated for 30 minutes with tyrothricin before activation with streptokinase.

TABLE IV. Effect of Tyrothricin on Plasmin Activity.

Tyrothricin	Units plasmin: Exp. 1	Exp. 2
None	64	104
50 μg	52	96

curred; streptokinase had already completed the activation of plasminogen and was no longer available for inhibition by tyrothricin. Since there is no inhibition of the proteolytic activity of the plasmin, tyrothricin must act on streptokinase, preventing the activation of the plasminogen.

A study also has been made of the effect of several antibiotics and chemotherapeutic agents on the activation of plasminogen by streptokinase, and on the lysis of the fibrin clot by plasmin. The compounds examined included penicillin, dihydrostreptomycin, polymyxin B, bacitracin, and sulfamerazine. All were tested at several concentrations of the active agent. No inhibition of either reaction was apparent with any of these compounds.

Summary. 1. Tyrothricin, or one or more of its components, reacts with streptokinase to prevent the activation of plasminogen and formation of plasmin. 2. Treatment of plasminogen with tyrothricin does not inhibit the activation of plasminogen by streptokinase. Plasminogen probably reacts with tyrothricin, preventing the inhibition of the added streptokinase by the antibiotic. 3. Tyrothricin does not inhibit the fibrinolytic activity of plasmin. 4. Penicillin, dihydrostreptomycin, polymyxin B, bacitracin, and sulfamerazine did not inhibit the activation of plasminogen by streptokinase nor the fibrinolytic activity of the plasmin.

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Received June 24, 1952. P.S.E.B.M., 1952, v80.

Toxicity of Mono Methyl Ether of Dipropylene Glycol (Dowanol 50B) in Studies on Auricular Fibrillation. (19699)

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A reliable method for the production of auricular fibrillation in intact animals would be useful in certain investigations. A recent report(1) on the consistent ability of the mono methyl ether of dipropylene glycol (Dowanol 50B) to provoke this arrhythmia in acute experiments on the anesthetized dog led us to inquire whether this compound could be used as a fibrillating agent in experiments repeated in a given dog on different days. The oral lethal dose was reported to be about 7.5 ml/kg (intravenous lethal dose not given) and the fibrillating dose 0.6 ml/kg intravenously or higher. Since recovery from sublethal dose administration was said to be complete, it seemed probable that Dowanol 50B could be used repeatedly as desired. We injected Dowanol 50B intravenously into 4 normal dogs in an attempt to determine whether the minimal dose producing auricular fibrillation in a given animal remains constant on different days. Such constancy of minimal dose has been reported to exist for repeated trials in acute experiments on a single day(1).

Methods. Five experiments were performed in 4 dogs. The animals each received 30 mg/kg pentobarbital sodium intravenously, and a tracheal catheter was inserted. Injections of Dowanol 50B were given rapidly into a foreleg vein while continuous recording of lead 2 was made with a direct writing electrocardiograph. Intervals of 10 to 20 minutes were allowed between injections. Because Dowanol 50B depresses respiration, dogs 1 and 2 were maintained on gentle positive blast respiration throughout, while dogs 3 and 4 were so maintained only after each injection, and were allowed to return to spontaneous respiration before a next injection was given.

Results. We found Dowanol 50B too lethal

for use in repeated experiments. Dog 1 (9.2 kg) was given 2 injections of 0.6 ml/kg each, without the production of fibrillation. With artificial respiration continued, 2 mg of methacholine chloride were given intravenously 17 minutes after the second injection, resulting in the appearance of auricular fibrillation with prolonged A-V block and later of markedly abnormal ventricular complexes. Death followed in about 5 minutes. A dose of 2 mg of methacholine chloride is rarely fatal in otherwise untreated dogs under pentobarbital anesthesia. Dog 2 (11.2 kg) received 5 injections totalling 3.0 ml/kg, the largest being 1.0 ml/kg without production of fibrillation. Two subsequent doses of 1.2 ml/kg each produced fibrillation. Death resulted after the respirator was disconnected one minute after the last injection. In dog 3 (12.7 kg) given 0.70, 0.75 and 0.70 ml/kg successively, fibrillation resulted after the second dose only. The animal died 15½ hours later and at necropsy showed gastric dilatation, pulmonary congestion and marked intestinal hemorrhage. In dog 4 (5.8 kg) administration successively of 0.17, 0.35, 0.69 and 0.52 ml/kg resulted in the production of auricular fibrillation only with the last 2 doses. The animal remained anesthetized for about 36 hours and thereafter had marked ataxia and cough for a week. In a second experiment 16 days later the minimal fibrillating dose was 1.1 ml/kg, determined after a total of 3.5 ml/kg had previously been given and after a dose of 1.0 ml/kg had failed to produce fibrillation. The animal died 2 days later without recovering from Dowanol anesthesia, 4.6 ml/kg total having been given during the last experiment.

Summary and conclusions. When administered intravenously to dogs anesthetized with pentobarbital, Dowanol 50B in adequate dosage produces auricular fibrillation, but in 4 dogs was found unsuitable for use in repeated experiments on different days, since it caused respiratory depression, prolonged anesthesia, disturbances of gait, intestinal hemorrhages and death. One animal surviving a first experiment required a significantly higher single dose and higher cumulative dose for production of fibrillation during a second experiment and succumbed following the latter.

We wish to thank the Dow Chemical Co. for a generous supply of Dowanol 50B.

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Received June 25, 1952. P.S.E.B.M., 1952, v80.

Effect of Liver Ischemia on Plasma in the Dog, as Measured by Electrophoretic Analysis.* (19700)

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Early attempts to study changes in the plasma proteins of dogs after hepatectomy were hampered by the short survival time of the prepared animals. Munroe and Avery(1) found no significant changes in the electrophoretic pattern of dog plasma, in periods up to 7 hours after hepatectomy. Lewis, Page, and Reinhard(2) were able to obtain plasma samples up to 16 hours after the operation. They reported but small changes. The total plasma protein fell, and a decrease in the β-globulin accounted for much of this change. We know of no reports of electrophoretic studies on plasmas of dogs with reduced hepatic circulation. Although Eck fistula dogs have been studied from a hematological viewpoint, there is little known about the electrophoretic patterns of their plasmas.

Our studies have been made on dogs which, strictly, were not Eck fistula dogs, although a portal-caval anastomosis and ligation of the portal stem close to the liver had been carried out in all of them. To obtain a maximum reduction of circulation in their livers, the dogs were subjected to a 3-stage operation in which, ultimately, all of the named vessels

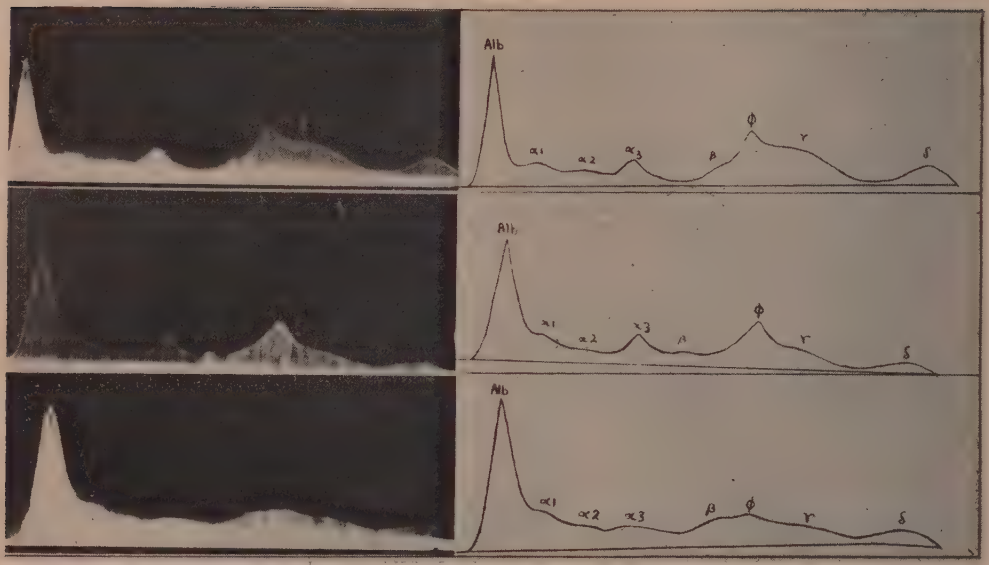

FIG. 1. Tracings of electrophoretic patterns of dog plasma, 2-3 months after stage 2. Top, dog A22; center, dog A9; bottom, normal dog.

which supplied the liver were completely tied off. The organ continued to live on collateral blood supply alone(3).

After the first and second stages of surgical preparation, the hepatic artery and all its branches are completely tied off, and the portal blood is in part diverted from the liver by a partial Eck fistula. This fistula consists of a portal-caval anastomosis and a ligation of the portal vein below its pancreatico-duodenal tributary. Venous blood from the pancreas, duodenum and stomach can still flow into the liver through the pancreatico-duodenal vein.

In the third stage of the operation, the pancreatico-duodenal vein was ligated, with the result that portal blood was totally diverted from the liver. The hepatic circulation in these dogs is now maintained mostly by arterial collaterals from the left gastric and phrenico-abdominal arteries. They supply the liver with arterial twigs which penetrate through the coronary ligaments and subserously through the hepato-duodenal ligament.

For the electrophoretic studies, 3 ml of heparinized plasma from the experimental animals were diluted up to 12 ml with barbiturate buffer, of pH 8.6 and ionic strength 0.1. The diluted plasma was dialysed against one liter of buffer for 18-20 hours at 0°C. Analyses were made in the standard analytical cell of the Aminco-Stern electrophoretic apparatus. With a potential gradient of 5.1-7.4 v/cm, satisfactory resolution of the plasma proteins took place in 60-90 minutes. Ascending patterns were analyzed to determine the relative concentrations of the various proteins. Total protein was determined by the micro-Kjeldahl method.

Observations and results. Significant changes in the electrophoretic pattern appeared only after the first and second stages of the surgical preparation had been completed. The plasma of such dogs showed a slight decrease in the albumin fraction and an increase in the sum of the $\beta + \phi + \gamma$ fractions. (Fig. 1 and Table I). The values of albumin recorded as normal in Table I may appear low, but this is found to be the average value for dogs on a "fox chow" diet. The A/G ratio is lower than normal.

In 3 other dogs (Fig. 2 and Table I) the plasma was analyzed 2 to 3 weeks after the third stage of the operation. The albumin is lower in these dogs than after the second

TABLE I.

	Albumin	α_1	α_2	α_3	β	ϕ	γ	A/G	Total protein, g %
Normal	39.2	11	7.3	11.9	——30.7——			.64	7.66
A9	36.7	—13.9—		11.1	3.2	—34.9—		.58	5.7
A22	27.6	9.7	6.8	9.7	——46.2——			.38	6.13
A30	24.4	—14.5—		9.8	——51.4——			.32	5.55
A49	9	5.8	7.1	8.3	——69.8——			.099	6.6
A50	18.6	15.6	4.5	12.6	——48.7——			.23	5.94

stage, and much lower than normal. The corresponding increase in globulin seems to have occurred in the $\beta + \phi + \gamma$ fractions. The A/G ratio is seen to be very low—dropping to 0.099 in the case of dog A49.

Analyses of plasma samples obtained several months after the different operative stages showed the following results, which are also summarized in Table II.

Dog A50 (see also Table I), 6 months after stage 3, has not recovered a normal albumin value (Fig. 3), but instead, the albumin has dropped slightly.

Dogs A35, A46, A48, 5 to 6 months after stage 3 (Table II), have more normal values of albumin. Apparently the collateral circulation has increased to assure normal protein production by the liver. It might be noted that Dog A48, though in a precarious state of nutrition and menaced by slight bouts of meat intoxication, still has an almost normal albumin level. It should be mentioned that all dogs have been maintained on a constant diet at all times.

That the condition of the dogs is at least as important as the time factor is seen from a

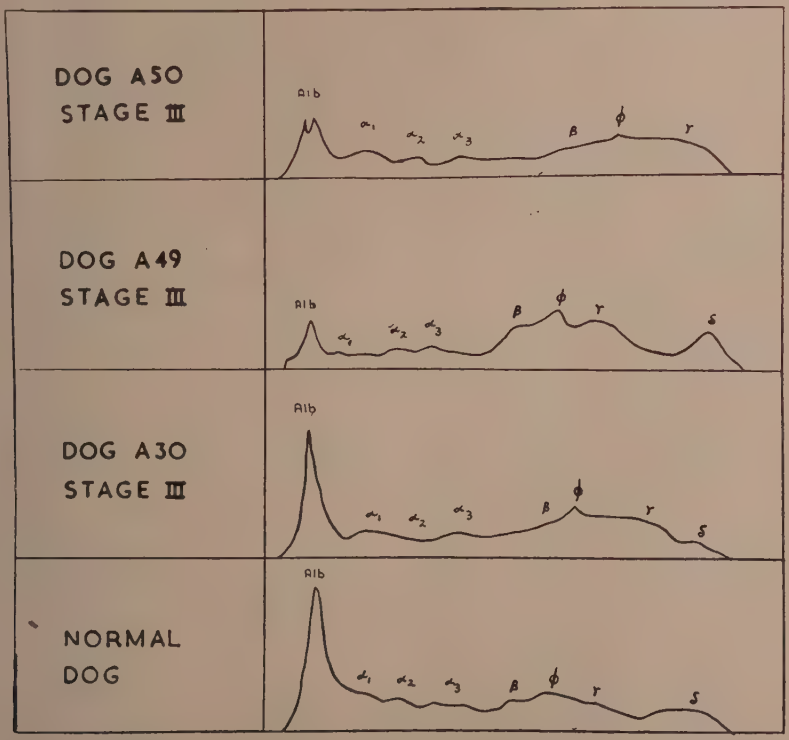

FIG. 2. Tracings of electrophoretic patterns of dog plasma, 2-3 weeks after stage 3.

TABLE II.

Run	Dog	Albumin	α_1	α_2	α_3	β	ϕ	γ	A/G
168	A50	15.7	3.5	6.3	13.4	37.7		23.4	.28
169	A35	41.1	10.4		12.7	6.2	18.3	11.2	.70
170	A46	35.1	12.6	6.4	6.7	11.3	19.9	12.1	.54
178	A58	40.7	20.4		12.2		26.2		.69
179	A48	34.6	11		21.6		32.6		.53
182	A55	28.6	10.6		22.3		37.8		.40
219	HC34	41.2	3.9	9.7	11.7	8.4	13.3	11.7	.70
207	HC28	37.7	14.2		16.2	7.9	13.5	10.7	.60
209	HC27	31.7	15.2		20	9.9	12.9	10.3	.46

consideration of the results after stage 2.

Dog A58, 6 months after stage 2, has a normal albumin level, whereas Dog A55, 7 months after stage 2, has a low albumin value (Fig. 3 and Table II). However, this latter dog had a very difficult recovery after stage 1, complicated by perforation of the gall bladder and generalized peritonitis. Although the recovery after the second stage was uncomplicated, still the animal had a prolonged convalescence with poor food intake.

Dog A9, 2 months after stage 2, had a normal albumin level whereas Dog A22, 3 months after the same operation, had a low albumin value (Table I). This dog went through several severe bouts of "meat intoxi-

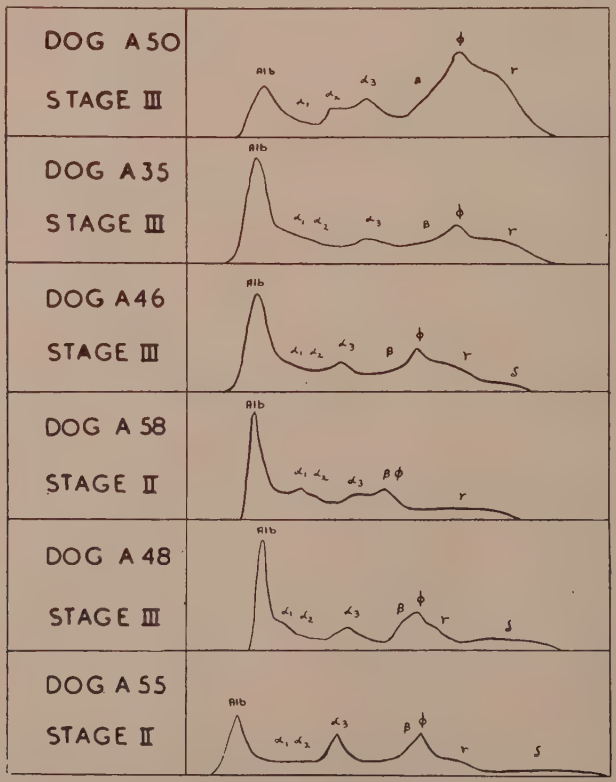

FIG. 3. Tracings of electrophoretic patterns of dog plasma, 6-7 months after stages 2 and 3.

cation" from which it recovered with difficulty. There is the impression that massive doses of vit. A were of some help. (This therapy was suggested by the observation of signs of vit. A deficiency, such as a scaly skin, sublingual ulcers, blindness, reduced sensitivity of the skin, etc.).

Two dogs (HC-27 and HC-28, Table II) in a state of hepatic coma produced by ligation of the common hepatic artery, combined with a classical Eck fistula(4) showed albumin percentages of 31.7 and 37.7, *i.e.*, not markedly low values. One animal, HC 34 (Table II), which had recovered from hepatic coma and was still alive after 13 months, showed a normal pattern.

Summary. Successive surgical interference with the blood supply to the liver is followed by a decrease in the total proteins. The greater the interference with the circulation, the lower is the albumin level. The higher globulin level is due to an increase in the $\beta + \phi + \gamma$ fractions. It is suggested that a longer lapse of time after the experimental reduction of the blood supply permits the establishment of an adequate collateral circulation and tends to reestablish the normal production of proteins by the liver. In acutely induced fatal hepatic coma no large changes in the electrophoretic patterns of the dog plasmas were observed during the short survival time.

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* This work was partially supported by a grant from the National Research Council of Canada.

Received June 26, 1952. P.S.E.B.M., 1952, v80.

Isolation of *M. tuberculosis* by Inoculation of the Yolk Sac of Embryonated Eggs.* (19701)

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Previous investigators(1,2) have demonstrated that *M. tuberculosis* proliferates with great rapidity in the yolk sac of embryonated eggs. These observations were made on embryos inoculated into the yolk sac with pure cultures of the microorganism. Careful search of the literature gives no indication that the yolk sac has been utilized for the rapid isolation of *M. tuberculosis* from materials collected from patients for the purpose of a more rapid laboratory diagnostic procedure. Investigations which are being performed in this laboratory indicate that inoculation of the yolk sac of chick embryos provides a rapid means for establishing the etiological diagnosis with materials from patients with suspected tuberculosis. A preliminary report of the results obtained thus far appears to be warranted.

Methods. Age of embryos and method of inoculation. Embryos of 5 to 8 days preliminary incubation are suitable. The yolk sac is inoculated through a small drill hole in the shell by means of a syringe and 20- or 22-gauge needle. After injection the drill hole is covered with melted paraffin.

Preparation preliminary to inoculation of materials tested. Only such materials collected from patients were tested in which a thorough search by the established methods, including concentration procedures, failed to reveal the presence of acid fast bacilli by microscopic examination. Six embryos each were inoculated into the yolk sac with each specimen.

Spinal fluid. Spinal fluid collected under aseptic precautions requires no preliminary treatment. The amount of fluid available determines the size of the inoculum which can vary from 0.2 to 0.5 ml per yolk sac.

Pericardial and pleural exudates. A preliminary test by inoculating 0.2 ml amounts

* Aided by grants from the Eli Lilly Co.

on a blood agar plate and into thioglycollate broth is run to assure bacteriological sterility. The size of the inoculum varies from 0.2 to 0.5 ml per yolk sac. Bacterial contaminants are controlled by the addition of appropriate antibiotics.

Gastric washings and sputum. These are digested in equal parts 4% NaOH at 37°C until all mucus and visible particles are dissolved and then centrifuged at 2000-3000 r.p.m. for 30-60 minutes. The supernatant is discarded and the sediment is carefully titrated to normal with 6% H_2SO_4. The sediment is then suspended in 1 to 2 ml of saline which contains 1000 units of penicillin per ml. Usually 0.1 to 0.2 ml of this suspension per yolk sac is used as inoculum.

Tissues and exudates. Tissues are ground with sterile alundum and suspended in saline containing 1000 units of penicillin per ml. Exudates too thick for aspiration are diluted in saline containing penicillin. 0.1 to 0.2 ml amounts are inoculated into each yolk sac.

Other materials. No opportunity for testing urine or stool samples has thus far presented itself. The sediment from the centrifuged urine specimens should present no difficulty. The presence of Gram negative bacterial contaminants may be controlled with the addition of chloromycetin. Stool specimens would require the judicious addition of antibiotics.

Incubation and examination of inoculated embryonated eggs. Inoculated eggs are incubated at 35 to 37°C. They are candled daily and embryos which die during the first 4 days are discarded. Beginning the fourth day after inoculation one embryo from each group is examined daily. The yolk sac is transferred with aseptic precautions to a sterile Petri dish.

Demonstration of acid fast bacilli in the yolk sac. Approximately 1 ml of the yolk is transferred to a 2.5 x 10 cm test tube and 20 ml of 15% phenol is added. The tube is sealed with a rubber stopper, shaken vigorously for 5 minutes and allowed to stand for at least 2 hours. By means of a capillary pipette a small amount of the fatty layer at the surface is carefully removed and smeared extremely thinly on the surface of one or more chemically clean slides. After drying and heat fixation the smears are stained by the Ziehl-Neelsen method and examined microscopically for the presence of acid fast bacilli. In most instances preparations made in this manner from the yolk sacs on the 4th day following inoculations contain typical acid fast bacilli in abundance.

Identification of the acid fast microorganisms as M. tuberculosis. Tuberculin negative healthy guinea pigs were inoculated into the groin and intraperitoneally with 0.5 to 1 ml from at least one yolk sac from the group of embryos used for each test. Three weeks later they were again tuberculin tested. Positive reactors were sacrificed and the presence of typical acid fast bacilli demonstrated in smears from the lesions.

Results. A total of 50 specimens of various types were subjected to yolk sac inoculation. Of these 39 were derived from patients with various types of tuberculous infection. Eleven specimens were obtained from known negative controls. Acid fast bacilli were demonstrable within 4 to 6 days after inoculation in every instance in which positive results were obtained. In each of these cases the acid fast bacilli demonstrated in the yolk sac cultures have proven to be *M. tuberculosis* by guinea pig inoculation. Acid fast bacilli have not been demonstrable in yolk sacs inoculated with materials from the negative controls. Guinea pigs injected with yolk from embryos inoculated with the control material have developed no evidence of tuberculosis. Table I summarizes the results thus far obtained.

Discussion. The yolk sac of the developing chick embryo apparently provides exceptionally favorable nutritional and environmental conditions for the rapid proliferation of *M. tuberculosis.* Within 4 to 6 days after inoculation acid fast bacteria are readily demonstrable from specimens in which these microorganisms are not demonstrable by direct examination. By this means the time period for detecting *M. tuberculosis* is shortened considerably as compared with that required by the established cultural methods and by animal inoculation.

The method has been found to be especially valuable in establishing the diagnosis

TABLE I. Results of Inoculation of Chick Embryo Yolk Sac for the Demonstration of *M. tuberculosis.*

Type of specimen		No. tested	Acid fast bacilli in yolk sac Pos.	Neg.	Inoculation of yolk into guinea pigs Pos.	Neg.
Spinal fluid	Tests	13	13	0	13	0
	Controls	6	0	6	0	6
Sputum	Tests	7	7	0	7	0
	Controls	0				
Gastric washings	Tests	5	5	0	5	0
	Controls	2	0	2	0	2
Pleural fluid	Tests	6	6	0	6	0
	Controls	2	0	2	0	2
Peritoneal fluid	Tests	3	3	0	3	0
	Controls	0				
Pericardial "	Tests	1	1	0	1	0
	Controls	0				
Tissue	Tests	4	4	0	4	0
	Controls	1	0	1	0	1
Total tests		39	39	11		
Controls		11				

in cases of tuberculous meningitis, tuberculous pericardial, pleural, and peritoneal effusions. Limited experience thus far indicates its usefulness in following the effect of therapy in cases of tuberculous meningitis.

No attempts have as yet been made in this laboratory to determine whether or not the method can be adapted to assess the effect of therapeutic agents. It also may prove to be useful in determining the development of drugfastness of strains of *M. tuberculosis.*

These investigations are being continued in order to assess more accurately the usefulness of this method as a practical laboratory procedure for the more rapid etiological diagnosis of tuberculosis infections.

Conclusions. Inoculation of the yolk sac of 5- to 8-day embryonated eggs provides a rapid method for demonstrating the presence of *M. tuberculosis* in materials collected from patients with suspected tuberculous infections. Acid fast bacilli are readily demonstrated in the yolk of embryos 4 to 6 days after inoculation.

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Received June 30, 1952. P.S.E.B.M., 1952, v80.

Influence of Niacin and Priscoline on Ketonuria, Liver Glycogen and Liver Lipid.* (19702)

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It has been established that when niacin is given in relatively large amounts to diabetic rats or normal fasting rats a marked ketonuria is exhibited by many of the animals(1,2).

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association and the Central Scientific Fund, State University of Iowa. The niacin was supplied through the kindness of Hoffmann-LaRoche, Inc., the priscoline through the kindness of Ciba Pharmaceutical Products, Inc.

TABLE I. Urinary Acetone Bodies, Liver Lipid and Glycogen in Rats Fasted for 5 Days. 12 animals in each group.

Treatment	Terminal wt, g	Urinary acetone bodies, mg/24 hr	Liver glycogen, mg %	Liver lipid, g %
Saline control	245	1.96 ± .5*	230 ± 35	7.71 ± .5
35 mg niacin daily	237	10.30 ± 1.9	463 ± 44	8.50 ± .4
20 mg priscoline daily	232	8.74 ± 2.7	90 ± 19	8.73 ± .8

* Stand. error.

Recently, it has been pointed out that priscoline also has a ketogenic effect in fasted rats (3). The question arises as to whether there may be alterations in liver fat or glycogen during this ketonuria. It has been shown that when an animal is in ketosis the liver usually has reduced glycogen levels(4), but Beringer(5) observed that the level of liver glycogen cannot always be correlated with the amount of ketone bodies formed. Also, total liver fat may be increased during a ketonuria but this is probably not obligatory(6,7).

In the present studies an attempt has been made to determine if there is any *correlation* between liver changes and the ketonuria induced by niacin or priscoline. This work has been briefly described in a preliminary note(3).

Methods. Thirty-six adult female rats of the Long-Evans strain were divided into 3 groups and fasted for 5 days. During the fasting period one group received daily intraperitoneal injections of 35 mg niacin in 7 cc physiological saline, another group 20 mg of priscoline in saline, while the third group was injected with saline alone. Urinary acetone bodies were determined for 24-hour samples on the third and fifth days of fasting by the gravimetric method of Van Slyke(8).

At the termination of the experiment the rats were anesthetized with sodium amytal and liver samples were taken for chemical and histochemical studies. Liver glycogen was determined by the method of Good, Kramer, and Somogyi(9) and histochemically by the periodic acid leuco-fuchsin method. Total liver lipid was determined by extracting homogenized tissue with alcohol-ether, evaporating the solvent and weighing the residue. Histochemically the lipid was demonstrated by an osmic acid procedure. The liver was fixed for 24 hours in Flemming's solution and then transferred to 2% osmic for 48 hours. This procedure more clearly delineated the fat than the Sudan-Black method. No counter stains were used.

Results. The saline injected group of rats excreted only small amounts of acetone bodies on the 5th day of their fast (Table I). The animals receiving niacin or priscoline, however, had increased levels of urinary ketone bodies. The priscoline group exhibited the largest variation in their ketogenic response.

There were quantitative differences in liver glycogen values in the 3 groups of rats. The glycogen values for the saline group were relatively high considering the animals had been fasted for 5 days. The niacin injected group, however, had higher levels and the priscoline injected much lower levels than the saline controls (Table I).

It was thus of interest to know what the histochemical preparations for liver glycogen would show as far as amount and distribution is concerned. The saline injected controls had normal glycogen distribution. Most of the glycogen was present in cells in the region of the central veins (Fig. 1) and with the exception of a few cells adjacent to portal triads, the rest of the liver was relatively free of glycogen. The liver glycogen of the priscoline injected rats was much less than seen in the controls and although the photomicrograph (Fig. 3) shows practically no glycogen, in some instances there was more than is represented in this picture. The niacin injected rats showed a remarkable amount of liver glycogen, similar to that found in non-fasting animals. Furthermore, the distribution of the glycogen droplets was somewhat different than that ordinarily observed since they were quite

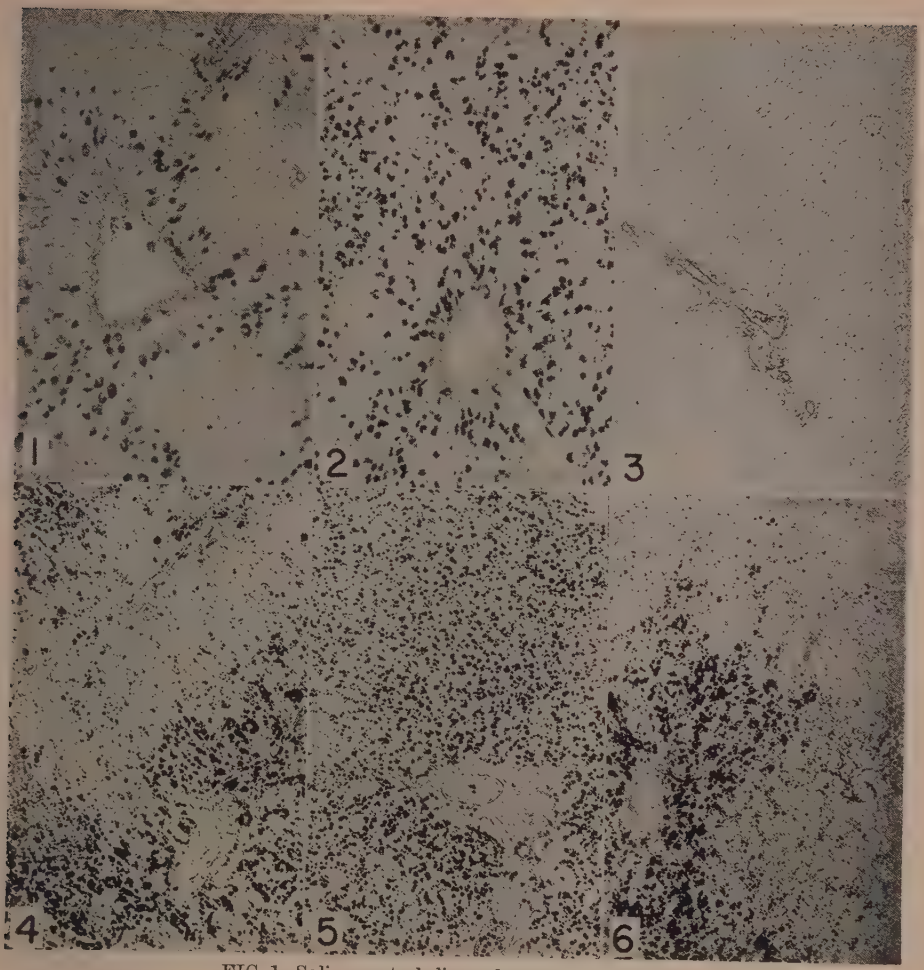

FIG. 1. Saline control, liver glycogen 154 mg %.
FIG. 2. Niacin injected, liver glycogen 560 mg %.
FIG. 3. Priscoline " " " 42 mg %.
FIG. 4. Same liver as Fig. 1, liver lipid 7.49 g %.
FIG. 5. " " " " 2, " " 7.00 g %.
FIG. 6. " " " " 3, " " 7.31 g %.
(All figures × 125.)

evenly distributed throughout the lobules (Fig. 2).

Because of the differences in the amount and distribution of liver glycogen in the 3 groups of rats, it was of particular interest to study the levels and distribution of liver fat. Chemically the amounts of total liver lipid were similar in the 3 groups of animals (Table I). Moreover, in the histochemical preparations the liver lipid in the saline and priscoline injected animals had a similar distribution. The lipid was present, chiefly in the region around the portal triads and consisted of small, medium and large intracellular droplets (Fig. 4, 6). The small droplets were generally present in the periphery of the liver cells. In the niacin injected animals the liver lipid was present as only small and medium droplets but the droplets were quite evenly dispersed over the whole liver section (Fig. 5).

In the liver preparations of each group of rats some nuclei contained osmiophilic material. Whether this was lipid is not clear because it was not possible to reproduce this picture with Sudan-Black stain. There was no microscopic evidence of liver injury in any of the animals.

The question arises as to whether there is any correlation between the described liver changes and the presence of increased levels of urinary acetone bodies. There apparently is no such correlation because a ketonuria is present, on the one hand, in niacin-injected rats which have high levels of liver glycogen and, on the other, in priscoline-injected rats which have low levels of liver glycogen. Evidence that priscoline modifies carbohydrate metabolism is brought forth by Sasson(10) who showed that the insulin requirements of some human diabetics is reduced following the administration of priscoline.

Summary. When large amounts of niacin or priscoline were given daily to rats fasted for 5 days, an excessive excretion of urinary acetone bodies was noted in most of the animals. Niacin-injected rats had higher levels of liver glycogen, priscoline-injected lower levels. Total liver lipid was not altered by either drug. There appeared to be no correlation between the amount of liver fat or liver glycogen and the presence of excess ketone bodies in the urine.

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Received June 30, 1952. P.S.E.B.M., 1952, v80.

Intramuscular Cortisone Administration to Dogs. (19703)

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Most of the experimental studies dealing with the effects of cortisone administration to dogs refer to profound changes in electrolyte and water metabolism, but there is little information available as to the glycemia and the behavior of the glucose tolerance test curves during and after prolonged injection with cortisone. Mushett, Porter, and Silber (1) reported that daily subcutaneous treatment of 3 dogs with 10 mg/kg for a period of 2-3 weeks brought about a profound polyuria and polydipsia. Davis, Bass, and Overman(2) in an extensive study were able to demonstrate the remarkable influence of cortisone on the ionic balance of normal and adrenalectomized dogs. The present study was originally an investigation of the influence of cortisone upon the carbohydrate metabolism in normal dogs. However, it was found that large doses of cortisone may produce a picture which resembles diabetes insipidus rather than diabetes mellitus.

Material and methods. Five mongrel dogs, 3 males and 2 females of a weight between 9 and 12 kg were used in this study. The animals were confined in metabolic cages and a measured amount of commercial dog food was fed. The amount of urine, the specific gravity and the amount of consumed drinking water were measured every morning. Urine

TABLE I. Carbohydrate and Water Balance Before and After Treatment with Cortisone.

	Before treatment*						After treatment				
		Fasting	Max (24 hr)					Highest fasting blood	Max (24 hr)		
Dog No.	Wt., kg	blood sugar, mg %	Drinking water, cm³	Urine, cm³	Spec. grav.	Cortisone treatment, mg/day	Wt, kg	sugar, mg %	Drinking water, cm³	Urine, cm³	Spec. grav.
DS 11	12.7	78	760	620	1027	50 mg daily for 13 days Total: 650 mg	11	98	3000	2600	1007
DS 13	9	93	400	300	1040	200 mg 7 days 100 mg 7 days Total: 2100 mg	8.5	105	6200	5790	1007
DS 14	9	95	680	400	1035	100 mg 7 days 50 mg 7 days Total: 1050 mg	8	84	5580	5000	1008
DS 15	10.5	76	300	220	1050	50 mg 7 days 25 mg 6 days Total: 500 mg	9.5	99	420	390	1037
DS 16	12.6	90	180	210	1045	300 mg 4 days Total: 1200 mg	12.4	106	1000	900	1018

* Period of observation 4-6 days.

sugar was determined by "Clinitest Tablettes" (Ames Co., Inc.) and blood sugars by the method of Miller and Van Slyke(3). Fasting blood sugar specimens were usually taken every second day during the experiment and every morning during the first 4 days of cortisone treatment. Glucose tolerance tests were carried out using the method of intravenous injection; 1 g per 1 kg of body weight were injected and samples drawn after 3, 10, 20, 40, 60 and 90 minutes. Cortisone (Cortone Acetate "Merck") was administered intramuscularly and the daily total dose was divided into several injections of 50 mg each. In one dog 300 mg a day was to be injected; 150 mg was given intramuscularly in 3 injections, the remaining 150 mg was administered intraperitoneally.

Results. It is obvious from Table I that a significant elevation of the fasting blood sugar following cortisone treatment was not observed in any of our 5 dogs. Fig. 1 shows a glucose tolerance test curve before and after treatment with 300 mg of cortisone a day for 4 days. Both curves are normal. Similar glucose tolerance test curves were obtained also from the other 4 dogs. It is further obvious from Table I that a daily dose of 50 mg and more maintained for several days was sufficient to produce a remarkable polydipsia and polyuria which reached in some instances 10 times the normal values (Fig. 2 and 3). Our electrolyte balance study in such polyuric conditions needs further investigation; however, we are able to say that the concentration of sodium and chlorine in urine is decreased. Hence, all the cardinal symptoms of diabetes insipidus such as polydipsia, polyuria, low specific gravity and low concentration of NaCl in urine have been observed. The test of water deprivation to a "point of discomfort" was performed in 2 polyuric dogs. The dogs were fed the usual diet but water was withheld. The urine showed a specific gravity lower than 1010. After 9 hours the experiment was discontinued because of the dehydration of the animals. Dog DS 11 was treated with pituitrin for one day; 5 I.U. were injected subcutaneously at 10 A.M. and 6 P.M. The posterior pituitary hormone was able to restore almost normal conditions as far

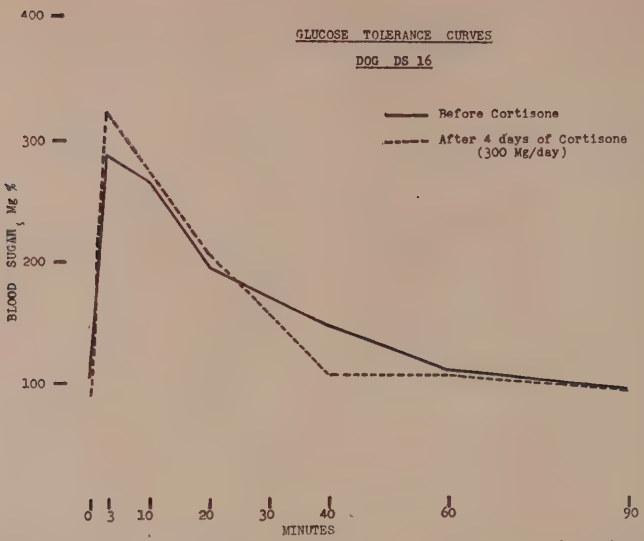

FIG. 1. Glucose tolerance curve before and after 1200 mg of cortisone.

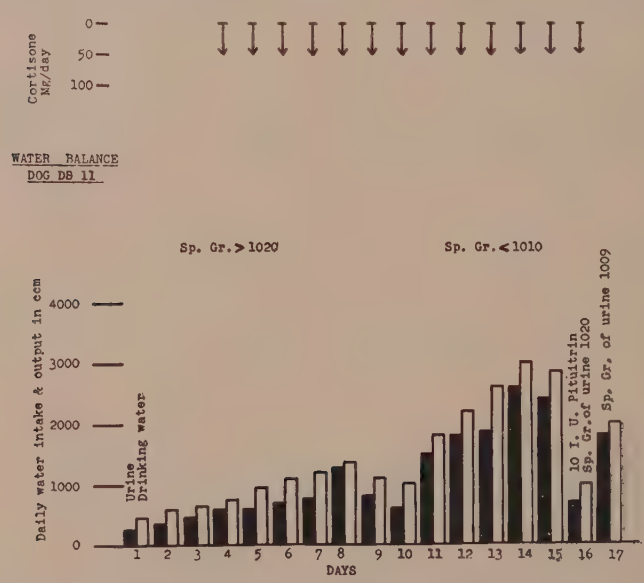

FIG. 2. Development of polydipsia and polyuria during cortisone treatment. Pituitrin was able to restore almost normal water balance.

as thirst, polyuria and specific gravity of urine were concerned (Fig. 2). Dogs DS 11 and DS 16 were followed for a certain time also after the cortisone treatment was stopped, while the dogs DS 13, 14 and 15 were sacrificed at the end of the injection period. The pancreas, kidney and hypophysis were examined histologically. In the dogs which were kept alive all the above mentioned symptoms of diabetes insipidus disappeared and normal conditions were established within 6-7 days.

Paraffin sections of the pituitary and of representative blocks (Bouin or formalin fixation) of the liver, kidney, pancreas, and ad-

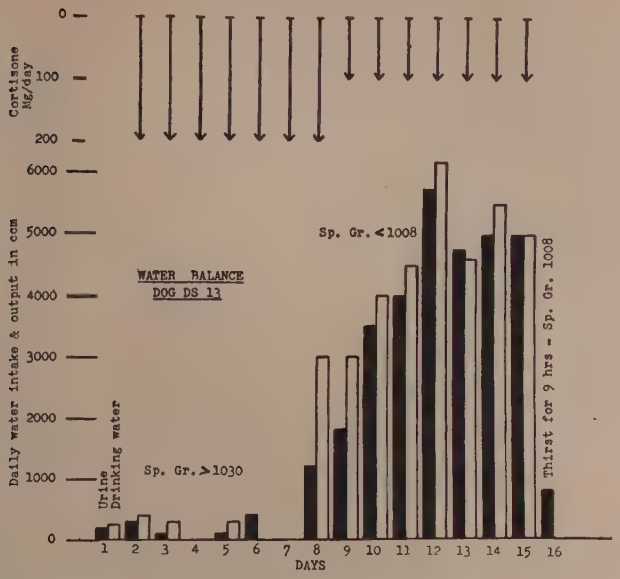

FIG. 3. Development of polydipsia and polyuria during cortisone treatment. Thirst for 9 hr had no remarkable effect upon the diuresis, and the specific gravity of the urine remained low.

renals were prepared in the usual manner and stained with hematoxylin and eosin. Sections of pancreas were specially stained to demonstrate the β-granules in the islets of Langerhans and examined by phase-contrast microscopy. Frozen sections of adrenals, kidneys and livers were stained by Wilson's technic(4) to demonstrate deposits of lipid. With one exception (see below) the tissues were free of pathological lesions, aside from terminal renal congestion and minimal fatty change in the liver. Deposits of stainable fat were present in the tubules of the kidneys from 2 of the dogs. Abundant lipid was present in the adrenal cortices of all animals, particularly in the zona reticularis. The intensity of β-cell granulation of the islets of Langerhans fell within normal limits.*

Discussion. The normal blood sugar values found in our experimental animals throughout the experiment on one hand and the production of the syndrome complex of diabetes insipidus on the other hand provide a good demonstration of the considerable overlapping between the group of "glucocorticoids" and "mineralocorticoids." The maintenance of a normal blood sugar level and the normal glucose tolerance test curves after large doses of cortisone are features quite similar to the response obtained by Fajans, Conn, *et al.*(5) with large doses of ACTH. An amount as high as 345 mg of ACTH per day injected for 4-6 days produced a typical diabetic glucose tolerance curve only in one out of 3 mongrel dogs.

A diabetes insipidus-like picture has also been produced in dogs by 11-desoxycorticosterone as reported by Ragan, Ferrebee, Phyfe, Atchley, and Loeb(6) and by Mulinos, Spingarn, and Lojkin(7). Britton and his coworkers(8-10) and Mulinos and associates(7), on the basis of their results, developed a theory that the adrenal cortex and the posterior pituitary are physiological antagonists in relation to certain phases of renal function. Thus, according to this theory, the increased water exchange of our dogs can be explained to a certain degree by a preponderance of adrenocortical activity to an extent which cannot be counterbalanced by the posterior pituitary, the end result being a diabetes insipidus-like picture.

*We are greatly indebted to Dr. W. Stanley Hartroft for making the histological examinations.

Summary. A syndrome closely resembling diabetes insipidus was produced in 4 mongrel dogs by daily injections of cortisone in doses of 50 to 300 mg a day. The period of hormone injection varied from 4 days to 14 days in different dogs. The polydipsia reached values as high as 6200 cc per day and the polyuria reached values as high as 5790 cc per day. Frequent fasting blood sugar determinations and glucose tolerance tests did not reveal any changes in carbohydrate metabolism.

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Received June 30, 1952. P.S.E.B.M., 1952, v80.

Agglutination of a Pure Strain of Mammalian Cells (L Strain, Earle) by Suspensions of Vaccinia Virus.* (19704)

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It is the purpose of this paper to record experimental results which demonstrate the ability of suspensions of vaccinia virus to agglutinate altered fibroblastic cells (L strain, Earle)(1). Heretofore, the reported evidence for agglutination of mammalian cells by viruses was limited to hemagglutination.

A variety of viruses have in common the ability to agglutinate erythrocytes(2-4). These viruses fall into two groups(5).† The first group is made up of the viruses of vaccinia, variola and ectromelia. These viruses possess hemagglutinins which are separable from the infectious viral particle. The second group includes the viruses of mumps, Newcastle disease and influenza. These viruses have hemagglutinins which are inseparably associated with the viral particle. It has been shown that viruses from the second group adsorb to cells other than erythrocytes. For example, influenza virus adsorbs to cells from ferret lung(6), and the virus of Newcastle disease has been shown to react with tumor cells to inhibit tumor growth(7). However, in those studies it was not possible to demonstrate that cells were agglutinated by the viruses.

During a study of the host cell-virus relationships between each of a variety of viruses and a strain of mouse cells cultivated *in vitro* (L strain, Earle), it was noted that a hemagglutinating virus which has a soluble hemagglutinin, vaccinia virus, caused cellular agglutination. This strain of cells has been maintained *in vitro* by Earle and coworkers since 1940(1). While being cultivated *in vitro*, the cells developed the capacity to produce sarcomas upon inoculation into mice(8). Since 1948, this strain of cells has had as its parent cell, a single cell(9). Cells from this strain may be considered as altered fibroblastic cells (10).

Materials and methods. Viruses. Three viruses were employed. Vaccinia virus was

* Aided by a grant from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council.

† For a review of the subject of hemagglutinins and hemagglutination, the reader is referred to reference 5.

TABLE I. Results of Viral Agglutination Experiments with Erythrocytes and L Strain Cells.

Virus	Exp. No.	Passage of virus*	Viral titer in rabbit skin	Viral hemagglutinin titer (reciprocal of final dilution of virus)	Agglutination of L strain cells at 36°C
Vaccinia	1	CA-3	10^{-5}	N.T.†	3/3‡
	2	CA-4	$10^{-6.5}$	"	6/6
	3	"	"	"	2/2
	4	CA-6	N.T.	128§	2/2
	5	CA-5	$10^{-8.3}$	16	3/3
	6	CA-6	N.T.	256	4/4
	7	"	"	"	2/2
	8	"	"	"	4/4
Mumps	1	A-1		256	0/2
	2	"		"	
	3	"		"	
Influenza type A	1	A-1		64	0/2
	2	"		"	
	3	A-?		2048	
	4	A-2		16384	

* "CA-3" signifies the third passage of vaccinia virus on chorioallantoic membranes. "A-1" indicates the first passage of mumps or influenza viruses in allantoic sacs of embryonated chicken eggs.

† N.T. signifies not tested.

‡ Numerator indicates number of cultural flasks which showed cellular agglutination; denominator signifies number of flasks inoculated with virus.

§ Discrepancies between titers of vaccinial hemagglutinin shown for passage CA-6 are within the limits of accuracy for the method employed since they represent only a 1 tube difference in results.

utilized as the supernatant fluid following centrifugation for 10 minutes at 1500 rpm of a 5% or 10% suspension of infected chicken chorioallantoic membranes. These membranes had been triturated by mortar and pestle in 5% dextrose in water. Vaccinia virus was quantitated for infectivity by the injection intracutaneously in rabbits of 0.2 ml aliquots of successive decimal dilutions of infected chorioallantoic membranes. The least amount of virus which produced lesions with induration, 5 mm or more in diameter in 4-7 days after inoculation was accepted as the titration or infectivity end point. Mumps virus was used as allantoic fluid from embryonated chicken eggs which had been infected 5 days previously. Infected allantoic fluid harvested after 48-72 hours of incubation at 36°C was the source of influenza virus, type A PR8 strain. Stock supplies of each virus (1 ml aliquots) were stored on dry ice for use during these experiments.

Cells. L strain. The L strain of mouse cells was kindly supplied by Dr. W. R. Earle. Cells were cultivated at 36°C on the glass wall of Porter flasks by the employment of a mixture of horse serum, 40%, chicken embryonic extract (1:1), 20%, and Hanks' solution, 40%. Cells were used for agglutination purposes after periods of from 1 to 8 days of cellular cultivation. The ability of the viruses of vaccinia, mumps and influenza to agglutinate L strain cells was determined by the addition of virus, 0.1 ml, to Porter flask cultures which contained 1.0 ml of liquid medium and sufficient cells to cover 80-100% of the wall of the flask. Cells were observed at 50x and 100x magnification for agglutination at 24 and 48 hours after the inoculation of virus. The results from agglutination tests which were attempted in test tubes with suspensions of L strain cells, were equivocal because spontaneous agglutination of the cells in the absence of virus frequently occurred.

Erythrocytes. Chicken erythrocytes from a single rooster were employed for hemagglutination tests, which were performed by the pattern method described by Salk (11). Dilutions of virus and a suspension of erythrocytes, 0.25%, were used in volumes of 0.4 ml each. The hemagglutination tests were read after 2 hours of incubation at 36°C for vaccinia virus and at room temperature for mumps and influenza viruses.

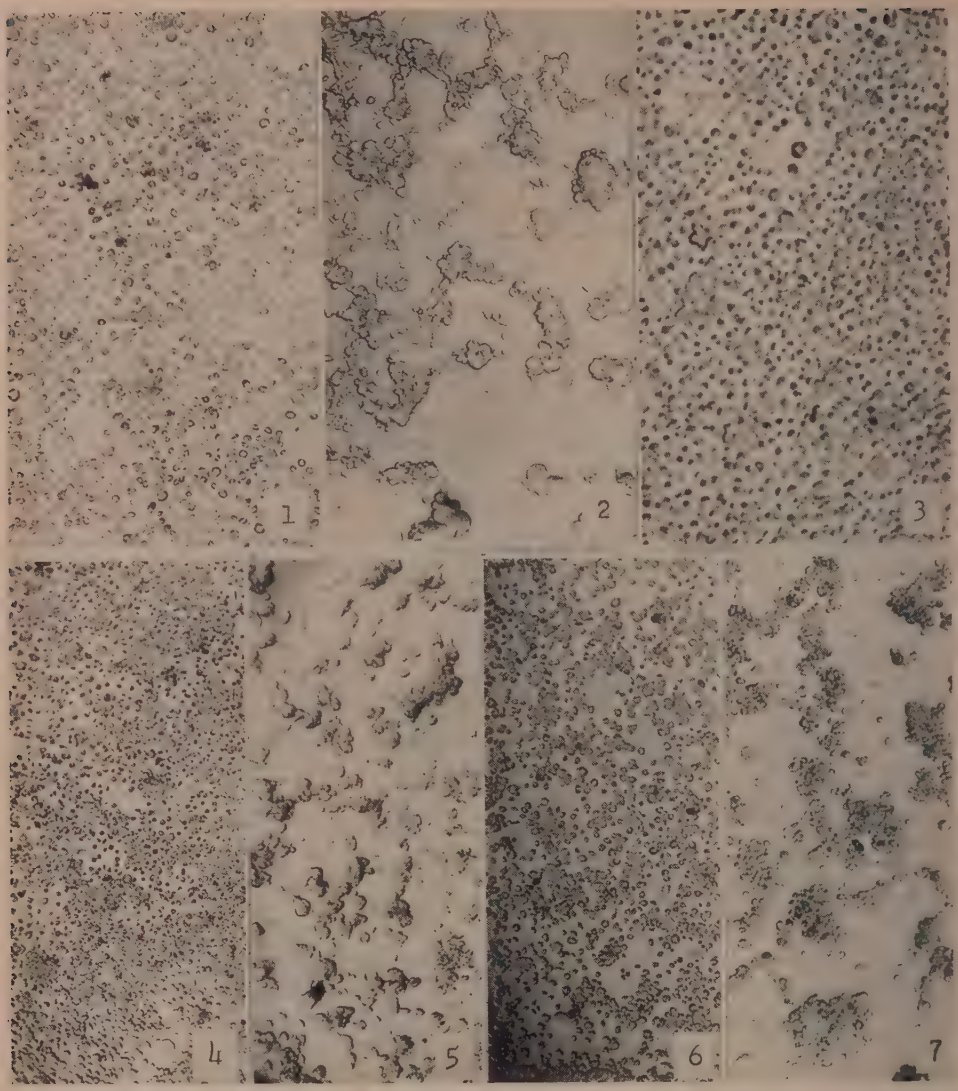

FIG. 1. Normal L strain cells; note the configuration and the even distribution of the cells. × 60.

FIG. 2. L strain cells showing agglutination 48 hr after the addition of a suspension of vaccinia virus. × 60.

FIG. 3. L strain cells from a culture 48 hr after the inoculation of a mixture of vaccinia virus and immune serum. Note the absence of cellular agglutination. × 60.

FIG. 4. L strain cells at 48 hr after the inoculation of mumps virus. × 45.

FIG. 5. Agglutination of the cells shown in Fig. 4 by vaccinia virus, added 5 days after mumps virus. This photograph was made 24 hr after the inoculation of vaccinia virus. × 45.

FIG. 6. L strain cells at 48 hr after the addition of influenza virus, type A, PR8 strain. × 60.

FIG. 7. Agglutination of the cells shown in Fig. 6 by vaccinia virus, added 5 days after influenza virus; at 24 hr after the inoculation of vaccinia virus. × 60.

Vaccinial antiserum. Pre-immunization serum was obtained from a normal rabbit. This animal was subsequently inoculated intracutaneously, for titration purposes, with 0.2 ml aliquots of decimal dilutions ($10^{3.3-9.3}$) of chorioallantoic membranes infected with vaccinia virus. Cutaneous lesions typical of vaccinia resulted from the lower 6 dilutions by 5 days after inoculation. Fifteen days after inoculation, serum was obtained from the rabbit. Both the pre- and post-immunization sera in small aliquots were stored at –20°C. Heat inactivation of complement was carried out prior to use of the sera for agglutination inhibition tests.

Results. Agglutination of L strain cells by suspensions of vaccinia virus. Table I presents the data from 8 experiments performed on separate occasions. It may be seen that L strain cells were agglutinated by vaccinia virus contained in each of 4 suspensions from different passages, CA^3-CA^6, on the chorioallantoic membranes of embryonated chicken eggs. Thus, each passage material readily agglutinated L strain cells even though in different passages, the infectious titers in rabbits ranged from $10^{5.0}$ to $10^{8.3}$, and the hemagglutination titers ranged from 1:16 to 1:256. Fig. 1 and 2 present the appearance of normal L strain cells and of L strain cells agglutinated by a suspension of vaccinia virus. For control purposes, it was determined that a 10% suspension of normal chorioallantoic membrane did not agglutinate L strain cells. It was observed that the extent of agglutination was not affected by the age of the cells in culture provided the cells had appeared normal microscopically at the time virus was added. For Exp. 7, L strain cells were observed for agglutination at 2, 4, and 7 hours, as well as at 24 and 48 hours. No agglutination was present at 2 and 4 hours. Slight agglutination was seen at 7 hours, and by 24 hours, marked agglutination was evident. Usually maximal agglutination existed by 24 hours after the inoculation of virus, but occasionally further clumping of the cells occured by 48 hours.

The presence in the liquid medium of horse serum and embryonic extract was not essential for agglutination of L strain cells to occur, since for Exp. 1 a synthetic medium (12) had been employed for maintenance of the cells during the agglutination experiment.

Titers of agglutinins for L strain cells and for chicken erythrocytes were determined simultaneously in Exp. 6. The final dilution of vaccinia viral suspension which agglutinated L strain cells was 1:32 and that which agglutinated erythrocytes was 1:256. An explanation of the difference in agglutinin titers is unknown. However, the cells obviously are dissimilar in origin, morphology and physiology, and the suspending medium for L strain cells, a mixture of horse serum, embryonic extract, and Hanks' solution, differed markedly from the saline employed for the hemagglutination tests.

For each agglutination experiment either with L strain cells or with erythrocytes, control preparations showed that no spontaneous cellular agglutination occurred in the absence of vaccinia virus.

No evidence was obtained to show that vaccinia virus multiplied in cultures of L strain cells. Vaccinia virus persisted in the liquid phase of L strain cultures for as long as 8 days after viral inoculation, but the titers were found to decrease gradually during that period of time.

Neutralization by immune serum of vaccinia viral agglutination of L strain cells. To establish that the presence of vaccinia virus, its soluble hemagglutinin or similar factor in suspensions of chorioallantoic membranes was causally related to the agglutination of L strain cells, vaccinial antibodies were used to inhibit agglutination. Serum obtained from a rabbit prior to immunization with vaccinia virus, and serum obtained following immunization were employed for viral inhibition tests which were done with both L strain cells and chicken erythrocytes. Equal quantities of serum and viral suspension were mixed and within 2-5 minutes, were added to L strain cells and erythrocytes. Table II presents the results from these agglutination inhibition tests. It is apparent for the dilutions of pre-immunization serum employed, that no inhibition of agglutination occurred for either L strain cells or erythrocytes. In contrast to the failure of pre-immunization serum to inhibit agglutination, the post immunization

TABLE II. Neutralization by Anti-vaccinial Virus Rabbit Serum of Vaccinia Viral Agglutination of Erythrocytes and Altered Mouse Fibroblasts (L Strain, Earle).

Cells	Final dilution of vaccinia virus employed	Reciprocal of final serum dilution — Pre-immunization					Post-immunization						
		32	64	128	256	C*	32	64	128	256	512	1024	C
Chicken erythrocytes	1:32 (4 hemagglutinating units)	+†	+	+	+	0	0	0	0	+	+	+	0
		24	48	96	192	384	C	24	48	96	192	384	C
L strain cells	1:24	+	+	+	+	+	0	0	0	±	+	+	0

* C signifies a control preparation of either the 1:32 dilution of serum for the hemagglutination tests or the 1:48 dilution of serum for L strain cells in admixture with test cells to rule out spontaneous agglutination.

† + denotes cellular agglutination; ±, slight agglutination; 0, no agglutination.

serum effectively prevented hemagglutination and L strain cellular agglutination. For example, L strain cellular agglutination by vaccinia virus was completely inhibited by a 1:48 dilution of the immune serum, and hemagglutination by a 1:128 dilution. Thus, evidence for a rise in the titer of vaccinial antibodies following immunization was demonstrable by the use of cellular agglutination of either L strain cells or of chicken erythrocytes. Fig. 3 shows the absence of cellular agglutination in a culture inoculated with vaccinia virus and immune serum.

Failure of the viruses of mumps, and influenza A to agglutinate L strain cells. For comparison with the results obtained with vaccinia virus, 2 viruses, mumps and influenza A, which agglutinate erythrocytes but do not form soluble hemagglutinins, were employed for experiments with L strain cells. The data that relate to 3 experiments with mumps virus and 4 experiments with influenza virus were included in Table I. It can be seen that although these viruses were potent hemagglutinating agents, they failed to agglutinate L strain cells in a total of 14 cultural flasks (Fig. 4 and 6) even though cultures were observed for as long as 7 days after viral inoculation. The possibility that the viruses of mumps and influenza were immediately inactivated by the horse serum-embryonic extract mixture employed as a nutritive medium for L strain cells, was excluded by the demonstration that the hemagglutinating properties of mumps and influenza A viruses, as well as vaccinia virus, persisted in cell free, horse serum-embryonic extract mixture for at least 24 hours. Attempts to agglutinate L strain cells at 22-25°C with mumps, influenza and vaccinia viruses have failed. However, L strain cells were readily agglutinated when a suspension of vaccinia virus was added at 36°C, to cultures which 1 to 5 days previously, had been inoculated with either mumps or influenza virus (Fig. 5 and 7).

Summary. Mouse "fibroblastic" cells from a pure strain of cells *in vitro* (L strain, Earle) were found to be agglutinated by suspensions of vaccinia virus, but not by the viruses of mumps and influenza A under the experimental conditions employed. The agglutination of L strain cells by suspensions of vaccinia virus was inhibited by vaccinial antibodies. The results of these experiments show that cellular agglutination by suspensions of vaccinia virus is not limited to erythrocytes.

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Received July 1, 1952. P.S.E.B.M., 1952, v80.

Effect of Adrenocorticotrophic Hormone on Tissue Distribution and Acute Toxicity of Beryllium. (19705)

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Many human cases of chronic pulmonary granulomatosis produced by inhalation of beryllium compounds have shown clinical improvement after treatment with adrenocorticotrophic hormone (ACTH)(1,2). These findings prompted the following study of the effects of ACTH on (a) the tissue distribution of injected radioberyllium, Be^7, and on (b) the survival of mice acutely poisoned with beryllium.

Methods. Beryllium was administered intravenously and ACTH intraperitoneally into young adult female CF #1 mice, weighing 21 to 27 g. In the tissue distribution studies, Be^7 was injected as the chloride either in tracer amounts (<0.001 μg/kg) or with sufficient carrier $BeSO_4$ to give each mouse approximately 0.3 mg Be/kg. This is a sublethal dose, the 14-day LD_{50} being 0.5 mg Be/kg. In the survival studies, mice in groups of 10 each received an LD_{95} of $BeSO_4$ in water, 0.7 mg Be/kg. Beginning immediately after the administration of beryllium, freshly prepared ACTH* in aqueous solution was given twice daily, in 1.25 mg amounts (equivalent to 2.5 mg of the standard ACTH), a dose schedule which will maintain a sustained eosinopenia and leucopenia in mice(3). In the survival studies, ACTH was given to the experimental group until death of the animals (2.5 days). In the distribution study, it was administered for 10 consecutive days and the mice were sacrificed on the 11th day when various tissues were removed for radioactivity analyses. Methods of assay were the same as described earlier(4).

Results. The data in Table I indicate that after 10 days of treatment ACTH had no significant effect on the distribution of radioberyllium in the presence of sublethal amounts of carrier $BeSO_4$. At the tracer level, however, there is a suggestion that ACTH leads to slightly increased elimination of Be^7, the probability values for the differences between femurs, residual carcasses, and total animals being 0.047, 0.036, and 0.06, respectively. These results extend those obtained in preliminary work(5) in which no change in tracer Be^7 tissue distribution was found at 3 days after a single intraperitoneal injection of 0.25 mg ACTH given either immediately or 42 hours after the administration of Be^7. The presence of carrier in sublethal amounts led to significantly greater retention of radioberyllium by the mouse than occurred with carrier-free Be^7, a result consistent with the findings in rats and rabbits(6).

Survival of mice given an LD_{95} of $BeSO_4$ was not affected by the ACTH treatment. The mortality curves for the treated and the untreated groups were almost identical, and the time to 50% death was 2.0 days for the former and 2.2 for the latter.

Discussion. To the extent that extrapolation from the mouse data is valid, these results indicate that ACTH probably is not responsible for any gross redistribution or excretion of Be in human cases of chronic berylliosis. It appears from available clinical data that there is no change in Be excretion during ACTH therapy of cases of chronic pulmonary granulomatosis produced by Be (1,7).

The fact that ACTH had no effect on acute experimental Be poisoning in mice, in which liver necrosis is the predominant pathological feature, while human cases of chronic pulmonary berylliosis are frequently benefited by this drug, reflects the differences between these conditions. These differences include route of administration or manner of exposure, the physical-chemical nature and the amounts of the beryllium compounds, and the lengths of time the tissues are exposed to beryllium. In the human cases ACTH is thought to be of benefit by producing a resolution of the granu-

* ACTH, Lot 212-103, 2 U.S.P. Units per mg, kindly furnished by the Armour Laboratories, Chicago, Ill.

TABLE I. Effect of ACTH on Distribution of Radioberyllium 11 Days after Injection of Be^7. % injected dose of Be^7.*

Tissue	Carrier-free Be^7		Be^7 + carrier	
	Be only	Be + ACTH	Be only	Be + ACTH
Liver	13.7 ± 1.7	10.4 ± 1.3	19.5 ± .3	17.8 ± .9
Femurs	2 ± .1	1.6 ± .1	1.9 ± .2	2 ± .04
Kidneys	.15 ± .02	.17 ± .03	.51 ± .06	.49 ± .02
Spleen	.58 ± .03	.47 ± .04	3 ± .2	2.8 ± .5
Lungs	.07 ± .004	.08 ± .004	.22 ± .02	.22 ± .03
Carcass	27.9 ± 1	24.3 ± .6	32.4 ± 1.4	31.7 ± 1.3
Total	44.4 ± 2.7	37.1 ± .8	57.5 ± 2	55 ± .7

* Each value is a mean based on 3 mice, except in the Be^7 + carrier control group, which consisted of 2 mice. Deviation is expressed as the standard error of the mean.

lomatous infiltration and possibly of the fibrosis(1,8) with resultant reduction of the alveolar-arterial difference in oxygen partial pressure(9).

The lack of effect of ACTH both on the survival of mice acutely poisoned with Be and on Be distribution suggests that there is no direct reaction between beryllium and either ACTH or any substance produced by ACTH activity. On the other hand, successful antidotal action against acute experimental beryllium poisoning in mice in the case of aurintricarboxylic acid is associated with the known ability of this substance to react chemically with Be under physiological conditions even though it produces no redistribution or increased excretion of Be(10,11).

Summary. Adrenocorticotrophic hormone (ACTH) had no appreciable effect on the distribution or retention of radioberyllium, injected into mice with and without carrier beryllium sulfate. The survival of mice acutely poisoned with beryllium sulfate was not influenced by ACTH.

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Received July 1, 1952. P.S.E.B.M., 1952, v80.

Protective Effect of Serotonin and of Para-Aminopropiophenone Against Lethal Doses of X-Radiation. (19706)

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In a previous publication(1) it was demonstrated in rats that pretreatment with pitressin or epinephrine afforded pronounced protection against total body x-irradiation. The protective effect was assumed to result from the production of a temporary tissue anoxia

TABLE I. Effect of Serotonin, PAPP and $NaNO_2$ on Survival of Rats after 880 r Total Body X-Irradiation.

Treatment (IP)	Time of treatment relative to x-radiation, min	Survival, 28-day		% methemoglobin	
		No.	%	Start of x-ray	Avg during x-ray period
Serotonin					
Control (H_2O)	5 before	3/55*	6		
20 mg/kg	"	30/31	97		
4 "	"	10/38	26		
PAPP					
Control (propylene glycol)	30 before	26/77	34	1.5	—
32 mg/kg	"	29/31	94	76.9	77.2
16 "	"	29/30	97	73.1	71.3
6 "	"	14/30	47	55.8	54.4
$NaNO_2$					
Control (saline)	10 or 30 before	5/44	11	2	—
60 mg/kg	30 "	9/30	30	52	54.7
60 "	10 "	2/23	9	25.4	42

* First number indicates survivals; following number indicates total animals, *i.e.* 3 survived out of 55.

by these agents. Further investigation of this pre-protection by vasoconstrictor agents was made employing serotonin (5-hydroxytryptamine), the vasoconstrictor agent present in blood platelets(2). In addition, to substantiate further the concept of the possible significance of the amount of oxygen in the tissue with regard to radiation effects, studies have been made on methemoglobinemia as a means of producing a reduced tissue oxygen tension.

Methods. Male Sprague-Dawley rats, weighing 270 ± 10 g, were irradiated in pairs, one serving as control, the other as a treated animal. Each pair was exposed to total body x-irradiation for 22 minutes in a single exposure. Radiation factors were: 200 kv, 6 ma, ½ mm Cu 1 mm Al filter, target distance approximately 29 cm, and 40 r/min., dosage rate measured in air.*

Either 1 or 5 mg of serotonin creatinine sulfate† in ½ ml of water were injected intraperitoneally 5 minutes before exposure. Methemoglobinemia was produced by the administration of either sodium nitrite or para-aminopropiophenone. Sodium nitrite was dissolved in isotonic saline to a concentration of 20 mg $NaNO_2$ per ml. Doses of 0.75 ml were administered intraperitoneally 10 or 30 minutes before exposure. Dosage levels of para-aminopropiophenone‡ were 8, 4, and 1.5 mg per animal, each administered intraperitoneally in 1 ml of propylene glycol. Blood methemoglobin levels were determined by a modification of the method of Evelyn and Malloy, as described by Storer and Coon(3). All controls received equivalent amounts of the appropriate solvent. Animals were housed in individual cages and weighed daily until death or termination of the experiment after 28 days.

Results. As would be expected with an 880 r dose in total body exposure, the majority of deaths occurred between 6 and 14 days. The effect of serotonin creatinine sulfate on survival rate after total body x-irradiation is shown in Table I. A dose level of 4 mg/kg body weight injected 5 minutes before exposure produced little, if any, protection; however, 20 mg/kg administered at the same interval prior to exposure produced a striking protective effect, as indicated by a survival rate of 97%, as compared with 6% survival for the control group.

Sodium nitrite and para-aminopropiophenone (PAPP) in varying doses were used to

* The authors wish to express their appreciation to the Radiobiology Department of this laboratory for assistance in the irradiation procedure.

† We are grateful to the Abbott Laboratories, Chicago, Ill., and to the Upjohn Co., Kalamazoo, Mich., for supplying us with samples of this preparation.

‡ We are grateful to the Dow Chemical Co., Midland, Mich., for supplying us with this preparation.

produce different blood levels of methemoglobin. Table I shows the methemoglobin level at the beginning of the experiment and the average level during the irradiation period. With PAPP, dosage levels of 32 and 16 mg/kg body weight produced average methemoglobin levels of 77 and 71% during the period that irradiated animals were exposed. Sixty mg/kg body weight of $NaNO_2$ 30 minutes prior to irradiation produced an average methemoglobin level of 54.7% during the period of exposure, compared to 54.4% resulting from a dosage of 6 mg/kg of PAPP. The survival rates obtained with these doses of PAPP and $NaNO_2$ are also shown in Table I. The two higher doses of PAPP, 32 and 16 mg/kg, gave excellent protection as evidenced by the 94 and 97% survival at 28 days. $NaNO_2$ (60 mg/kg) and PAPP (6 mg/kg) administered 30 minutes before exposure gave very nearly the same slight protection when the 28-day survival rates were compared with their respective controls. One group injected with $NaNO_2$ (60 mg/kg) 10 minutes before x-irradiation showed no protective effect with an average methemoglobin level of 42% during the period of exposure. The control animals in the PAPP experiments received 1 ml of propylene glycol 30 minutes prior to exposure and the survival rate in this group was higher than in the untreated or saline treated controls.

Discussion. The protective effect elicited by serotonin creatinine sulfate is assumed to lie in its vasoconstrictor property(2), producing a transient tissue anoxia in a manner similar to that of epinephrine(1). The possible influence of this agent on metabolism has not as yet been fully investigated.

Apparently there is a correlation between the degree of methemoglobinemia and protection against radiation. The protective effect of methemoglobinemia at certain concentrations may be due to the decreased supply of oxygen to the tissues, thus rendering them anoxic. The results with $NaNO_2$ and PAPP reported here agree with similar observations obtained with mice by Storer and Coon(3). The possibility that PAPP and $NaNO_2$ may affect the oxygen uptake of the tissue directly has to be taken into consideration. Cole, Bond, and Fishler(4) have reported that the mortality of mice receiving 600 r or 750 r single-dose whole body x-irradiation was reduced markedly following pre-irradiation intraperitoneal injection of $NaNO_2$ (100-125 mg/kg). They discuss the possibility that nitrite protection is mediated via its effect on catalase activity. The dose level of $NaNO_2$ per kg employed by these authors is higher than that used in the present investigation. However, it was found that 60 mg/kg was very close to the lethal dose for the rats employed. Unfortunately, the above authors did not carry out any methemoglobin determinations, permitting a comparison of methemoglobin levels with the degree of protection. The observation that administration of propylene glycol apparently exerts a slight protection is in agreement with similar observations of Salerno, Mattis, and Friedell(5).

The findings on the protective action of serotonin and PAPP, when administered prior to a lethal dose of x-irradiation, seem to substantiate the concept of a relationship between tissue oxygen tension and radiosensitivity. Alterations of susceptibility to the effects of radiation by changing oxygen tension in the tissues are probably due to the reduction in the formation of reactive decomposition products of water. In such a concept, cognizance should be taken, of course, of the effects of those decomposition products of water (such as the OH radical), which are formed on irradiation in the absence of dissolved oxygen.

In this connection, it may be opportune to refer to the observations of Bennett, Chastain, Flint, Hansen, and Lewis(6) that life span of rats receiving 600-1400 r whole body roentgen irradiation under anoxia was considerably shortened. This occurred in spite of the protection the anoxia gave against the lethal action of x-rays in the immediate post-irradiation period of 28 days. However, the main object of our investigation was to gain further insight into the mechanism of radiation injury and not so much to find protective means against radiation.

Summary. 1. The survival rate of rats, exposed to lethal x-ray dosage, was found to be significantly increased after pretreatment with

serotonin creatine sulfate (20 mg/kg) or para-aminopropiophenone (32 mg or 16 mg/kg). 2. The protective effect of these agents is assumed to be due to their property of producing a temporary tissue anoxia.

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Received July 3, 1952. P.S.E.B.M. 1952, v80.

Inhibition of Bacterial (*Escherichia Coli*) Modification of Erythrocytes. (19707)

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It is an established fact that certain viral and bacterial antigens may be adsorbed on red blood cells; this may result in hemagglutination (direct microbial hemagglutination), in modification of the erythrocytes or both. These modified red blood cells are specifically agglutinated by the homologous antimicrobial antibody (indirect microbial hemagglutination). It was shown recently from this laboratory(1,2) that boiled suspensions of *E. coli* serogroups 0111 and 055 as well as sterile broth culture filtrates are capable of modifying red blood cells from a variety of animal species. These modified red cells are agglutinated and, in the presence of complement, lysed by the group-specific *E. coli* antiserum. It was demonstrated furthermore that this agglutination and hemolysis is specifically inhibited by the homologous bacterial antigen. The experiments to be recorded in this communication revealed that certain materials inhibit the modification of red blood cells by *E. coli* antigen.

Material and methods. In addition to *E. coli* serogroups 055 and 0111 used in the previous experiments several strains of *E. coli* 026 were employed, since the latter serogroup, too, has been found to be associated with epidemic diarrhea of infants(3). For the modification of red blood cells boiled suspensions from Kolle flasks were used, as described previously(2). The microorganisms were suspended in the materials to be tested for inhibitory activity, namely, serum from man and various animals, human plasma fractions, bovine albumin (Armour), egg white, egg yolk, various fractions of rat liver, and, for control purposes, physiological saline solution. The human plasma fractions were obtained from Cutter Laboratories through the courtesy of Dr. F. F. Johnson and the rat liver fractions through the kindness of Dr. Charles U. Lowe. The mixtures were kept at room temperature for 15 minutes and then used for modification of red blood cells. The treated erythrocytes were washed 3 times in physiological saline solution. The hemagglutination and hemolysis tests were carried out as described in detail previously(2).

Results. It was found that a variety of human and animal sera, including sera from healthy adults and children, human cord serum, calf, beef, horse, sheep, chicken, duck and turkey sera in dilutions of 1:10 and 1:100 either completely or partially inhibited the modification of sheep red blood cells by *E. coli* 026 antigen. Quantitative experiments revealed that the more concentrated the *E. coli* antigen the more serum is required to produce inhibition of modification of red cells. The

TABLE I. Hemagglutination and Hemolysis by *E. coli* 026 Antiserum of Sheep Red Blood Cells Modified by Boiled *E. coli* 026 Antigen.

E. coli antigen diluted in	Titer of antiserum producing Hemagglutination	Hemolysis
Saline solution	1:1600	1:12800
Human serum (1%)	—*	—
Human plasma fractions (.1%)		
Albumin	—	—
II	1:1600	1:12800
III	1:400	1:6400
II + III	1:400	1:6400
III – 0	—	1:1600
III – 1	1:800	1:3200
III – 2	1:1600	1:12800
III – 2,3	1:200	1:1600
IV – 1	—	—
IV – 6	—	1:400
IV – 7	—	—

* — = No agglutination, no hemolysis with *E. coli* antiserum in dilutions of 1:100 and higher.

same results were obtained in experiments in which *E. coli* 0111 and 055 and red blood cells of man and other animal species were employed. Furthermore, egg yolk suspensions (10% to 0.01%) in physiological saline solution had like effects. In contrast, egg white (in dilutions up to 10%) was essentially ineffective. Rat liver fractions (nucleus-free cytoplasm (1 ml = 0.2 g of liver), nuclei (1 ml = 0.5 g), mitochondria (1 ml = 0.5 g), microsomes (1 ml = 0.5 g)), also inhibited the modification of sheep cells.

In view of the inhibitory effects of human serum it was deemed of interest to determine the activity of various human plasma fractions. In these experiments boiled *E. coli* 026 antigen (diluted 1:50) was used to yield approximately 2 minimal hemagglutinating units; the antigen was diluted in the available plasma fractions (0.1%), human and chicken sera (1%) and in physiological saline solution. Blood cells modified by this antigen were specifically agglutinated—and in the presence of complement lysed—by *E. coli* 026 B6 antiserum but not by *E. coli* 055 and 0111 antisera. The results are summarized in Table I. It can be seen that certain fractions prevented hemagglutination and hemolysis completely, whereas others were minimally effective or completely ineffective. Since, as shown previously(2), *E. coli* antiserum produces hemolysis of sheep cells in far higher titers than hemagglutination, it is not surprising to find that some of the fractions inhibited hemagglutination to a greater extent than hemolysis. Commercially available human gamma globulin (up to 10%) was found to be ineffective.

This inhibitory effect takes place rapidly, since the inhibitor is in contact with the bacterial antigen for a total of less than one hour; the dissolved inhibitor is removed by repeated washing of the red cells. Continued contact of the inhibiting plasma fraction IV-1 for 18 hours at 4°C did not result in substantially greater inhibition than contact for less than one hour.

Regarding the heat stability of the inhibitory materials the following observations were made. Heating of the inhibitors in the concentrations used for one hour at 56°C did not reduce their activity. Boiling of human and animal sera (1%) for 10 minutes impaired their inhibitory activity but slightly and boiling for one hour only moderately. A substantial or complete loss of inhibitory activity occurred following boiling for 10 minutes of human plasma fractions IV-1, IV-4, IV-7, and albumin as well as bovine albumin and egg yolk (all in 0.1% concentration).

Since, as shown in repeated experiments, treatment of sheep cells with the above mentioned inhibitors (human serum (1%), human plasma fraction IV-4 (0.1%), sheep serum (0.1% to 10%), bovine albumin (0.3%), and egg yolk (0.1%)) did not interfere with their modification by subsequent treatment with *E. coli* antigen, the inhibitors must act either on the bacterial antigen itself or on the process of its adsorption on red blood cells. To determine which of the two possible modes of action actually takes place, the following experiments were carried out.

A boiled suspension of *E. coli* 026 was diluted 10 fold (vol. 10 ml) in 1) bovine albumin (3%), 2) human plasma fraction IV-1 (1%), 3) normal human serum (20%), and 4) physiological saline solution. The suspensions were kept at room temperature for 15 minutes. The bacteria were then washed 3 times with physiological saline solution;

TABLE II. Effect of Heat on Inhibitory Effect of Egg Yolk on *E. coli* Modification of Red Blood Cells.

E. coli antigen in	Titer of *E. coli* antiserum producing Hemagglutination	Hemolysis
Saline solution	1:1600	1:25600
Egg yolk (.1%)	—	1:200
Boiled egg yolk (.1%)	1:200	1:3200
Egg yolk (.1%) (mixture boiled)	—	1:100

— = No agglutination, no hemolysis with *E. coli* antiserum in dilution of 1:100 and higher.

separation of bacterial cells from the surrounding fluid was accomplished by means of a refrigerated centrifuge (International, model P R-1: r.c.f. at tip of tube calibrated to be 24,000; centrifugalization for 20 minutes). The sediment was suspended in 5 ml of physiological saline solution and then used for modification of sheep red blood cells. The experiment revealed that the bacteria treated with the 3 inhibitors—in contrast to the untreated bacteria—failed to modify the red cells, as indicated by absent hemagglutination and hemolysis by *E. coli* antiserum. These results suggest that the inhibitors act directly on the bacterial antigen.* This conclusion is substantiated further by the results of the following experiment. *E. coli* 026 antigen was diluted in 1) physiological saline solution, 2) egg yolk (0.1%), 3) egg yolk (0.1%) which had been boiled for one hour and 4) egg yolk (0.1%). All mixtures were kept at room temperature for 15 minutes. The latter mixture was then boiled for one hour to inactivate the inhibitor. The materials were used for modification of sheep red blood cells and the modified red cells were tested with *E. coli* 026 antiserum. The results are summarized in Table II. It is evident that boiled egg yolk is less effective as an inhibitory agent than the unboiled material and that egg yolk which had been allowed to act on the bacterial antigen and was subsequently boiled and thus partially inactivated had produced greater inhibition than was effected by boiled egg yolk.

Discussion. Bacterial hemagglutination and hemolysis are of considerable interest from several points of view. In the first place, direct bacterial hemagglutination may be used for the differentiation of closely related microorganisms, for example, *H. aegyptius* and *H. influenzae*(4). Secondly, indirect bacterial hemagglutination and hemolysis are useful tools for the serologist, as exemplified by the Middlebrook-Dubos test for the demonstration of antibodies against products of the tubercle bacillus(5). Thirdly, the possibility exists that these phenomena may take place *in vivo* and conceivably could be responsible for hemolytic anemia associated with bacterial infections, similar to the hemolytic process described to develop concomitant with certain viral diseases(6). The above reported experiments revealed that sera of man and various animals as well as certain, but not all, human plasma fractions inhibit the modification of red blood cells by *E. coli* antigen. Due to this inhibition these erythrocytes are not agglutinated or lysed by the homologous group-specific *E. coli* antiserum. Inhibition was also demonstrated with egg yolk and certain fractions obtained from rat liver. It has been shown furthermore that these inhibitors act mainly on the bacterial antigen and not on the red cells. This inhibition, then, differs from that due to receptor destroying enzyme interfering with viral hemagglutination.

This inhibition of *E. coli* modification of red cells is of interest also in connection with the previously reported(2) fact that unheated, in contrast to boiled, *E. coli* suspensions fail to modify red blood cells. It is conceivable that inhibitor is present in the unheated bacterial suspension which is destroyed by heating. Since the B antigen of *E. coli* interferes with bacterial O agglutination, it is possible that it is this antigen which also interferes with the modification of red blood cells. When purified B antigen becomes available, it will be of interest to determine its effects on *E. coli* modification of erythrocytes. The presence of inhibitors in various bacterial species may account for their inability to modify red blood cells. Further studies on the presence of red blood cell modifying antigens and their inhibitors in microorganisms are clearly indicated.

* The inhibitors do not interfere with bacterial agglutination.

It is of interest to point out that certain human plasma fractions (III-0, IV-1 and albumin) which inhibit bacterial modification of red cells also bring forth agglutination of red blood cells in the presence of incomplete Rh antibodies(7). Whether these 2 phenomena have an underlying mechanism in common remains to be determined.

Summary. 1. The modification of red blood cells by boiled suspensions of *E. coli* serogroups 026, 055 and 0111 is inhibited by human and animal sera as well as by egg yolk and various fractions of rat liver. Certain human plasma fractions in concentration of 0.1% (particularly fractions IV-1, IV-7 and albumin) are likewise effective, whereas others are not. 2. This inhibition is due largely to the action of the inhibitor on the bacterial antigen rather than on the red blood cell. 3. The significance of the results is discussed.

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Received July 3, 1952. P.S.E.B.M., 1952, v80.

XYZ Effect in Strain of Origin: EO771 Carcinoma in C57 BL/6 Mice. (19708)

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The xyz factors(1-11) are specific agents present in long-frozen or lyophilized tissue or fresh supernatant fluid from certain malignant tumors which, when injected, render the animal hosts more susceptible to the subsequent transplant of the same tumor. If the xyz factors are to affect the malignancy of spontaneous tumors in nature then it must be shown that each factor in transplanted tumors will influence the course of the same neoplasm in the inbred strain of origin. Prior experiments have shown that the EO771 factor would enhance the course of malignant disease in the C57 black strain(7,8) as well as in foreign strains of mice(5,6,11). It did not affect the course of transplanted tumor growth in C57 blacks for 3 other mammary cancers which arose in C57 black mice (755, 241-5, 241-16) nor did extracts of the other 3 tumors affect the course of EO771 in C57 blacks (7,8). This seemed significant, particularly since the EO771 xyz factor has been demonstrated in some concentration in the normal liver and kidney of the Jackson Laboratory strain of C57 blacks but not in normal spleen (10,11). Mammary cancers 755, 241-5, 241-16 had been carried in the National Cancer Institute strain of C57 blacks, and although the EO771 tumors used came from the Jackson Laboratory the experiments were carried out with the Carworth Farms strain of C57 black mice. The possibility arose that the diversity of C57 black strains used might have influenced the results. The present paper reports experiments on the effect of the EO771 xyz factor obtained from tumors inoculated in the Jackson Memorial Laboratory and tested on C57 BL/6 mice obtained from the same source.

Materials and methods. Fifty-two C57 BL/6 mice, 3-4 months of age, were obtained in one batch from the Jackson Memorial Laboratory in Bar Harbor, and were caged in 8 boxes of 6 or 7 mice in each.

Twenty-six mice (7 males and 19 females) were injected subcutaneously into the left

TABLE I. Rapidity of Tumor Growth and Mortality from Mammary Carcinoma EO771 in 52 C57 Black/6 (Jax) Mice, 26 of Which Had Been Pretreated with Frozen EO771 Tumor Tissue.

Exp. #	Mice (#, sex)	Breed	Treatment: Sr^x	Treatment: $Days^a$	Treatment: $Days^b$	Inoc. source	Median diam., cm*	Died	Med. day†	Mortality days
5069	6 ♀	BL	BL^1	49	32	BL^2	1.7	6	20	14, 19, 19, 21, 22, 25
5013	7 ♂	"	BL^1	49	32	"	1.9	7	18	14, 17, 18, 18, 19, 25, 41
5006	7 ♂	"	—	—	—	"	1.4	7	22	20, 21, 22, 22, 24, 35, 43
5082	6 ♀	"	—	—	—	"	1.4	6	26	21, 23, 25, 26, 29, 42
5075	7 ♀	"	BL^1	49	34	BL^3	1.5	7	22	14, 17, 20, 22, 24, 25, 28
5089	6 ♀	"	BL^1	49	34	"	1.8	6	21	15, 20, 20, 21, 21, 21
5095	7 ♀	"	—	—	—	"	1.3	7	23	23, 23, 23, 23, 24, 26, 36
5102	6 ♀	"	—	—	—	"	1.4	6	22	19, 20, 20, 23, 26, 30

* Median diameter of the tumors measured in 3 dimensions at 15 days.

† Median day of death for the group.

BL—C57 BL/6 (Jax) mice; Sr^x—breed of mouse from which EO771 tumor was taken for freezing; BL^1—C57 BL/6 (Jax) mouse; $Days^a$—length of time tumor kept frozen at 0°F; $Days^b$—interval between inj. of frozen tumor tissue and tumor transplantation; Inoc. source—source of EO771 tumor used for transplantation; BL^2 and BL^3—C57 BL/6 (Jax) mice from which EO771 tumor was taken.

groin with 0.1 cc of a 1-3 dilution of frozen tumor tissue (xyz factor) in normal saline (Groups 5069, 5013, 5075, 5089; Table I). No tumors or palpable reaction occurred at the site of the injections. The tumor tissue had been stored aseptically in 50% glycerin in normal saline for 49 days at 0°F and had been taken from a C57 BL/6 mouse inoculated with EO771 in the inbred nucleus at the Jackson Laboratory (all tumors from the Jackson Laboratory shipped through the courtesy of Dr. George Snell). The remaining 26 mice (7 males, 19 females) were controls, receiving no treatment prior to transplantation of viable tumor (Groups 5006, 5083, 5095, 5102).

The 52 mice were redivided into 2 groups of 26 mice in each so that each new group contained 13 experimental and 13 control mice. Two C57 BL/6 mice carrying EO771 tumor, received from the inbred nucleus at the Jackson Laboratory, were used for the subcutaneous transplantation of the 52 mice (Table I). The tumor tissue was prepared by mincing and grinding without sand with the addition of 3 parts of normal saline; 0.1 cc of the emulsion was injected subcutaneously into the left groin in one group and into the right groin in the other group. The transplants were successful and all 52 mice died from tumor growth with 14-42 days after inoculation. Measurements were taken of the growth in 3 dimensions twice weekly and the mortality was plotted in days after the tumor transplantation. The "volume" of the tumor was calculated for each mouse at 15 days by using the product of the diameters in 3 dimensions. (Three mice died on the 14th day).

Although all 52 mice died from tumor growth, the 26 mice pretreated with the nonviable frozen tumor tissue developed tumors which grew more rapidly and killed sooner than was the case among the 26 control mice (Fig. 1, 2). The mean volume of the primary tumor at 15 days was 3.26 ccm for the 26 controls (sum $X^2 = 428.6$, var. $m_x = 0.23$) and 6.62 ccm for the 26 mice pretreated with frozen tumor tissue (sum $X^2 = 1397.6$, var. $m_x = 0.40$). The difference of 3.4 ± 0.8 ccm was statistically significant ($t = 4.24$, $n = 50$, $P = 0.001$-). The mortality according to days after inoculation was significant when calculated by the method of Shear, Imagawa, Syverton, and Bittner(9). When 50% of the treated group had died only 13% of the controls were dead, and when 50% of the controls were dead 78% of the treated animals had succumbed.

Discussion. Since the first paper on the testing of xyz factors in the inbred strain of origin(8) Shear, Imagawa, Syverton, and Bittner have demonstrated that the xyz factor present in Z (C3H mice, Bittner) tumor 8352 is effective in influencing the course of the same neoplasm in the inbred strain of origin(9).

Summary and conclusions. Fifty-two C57

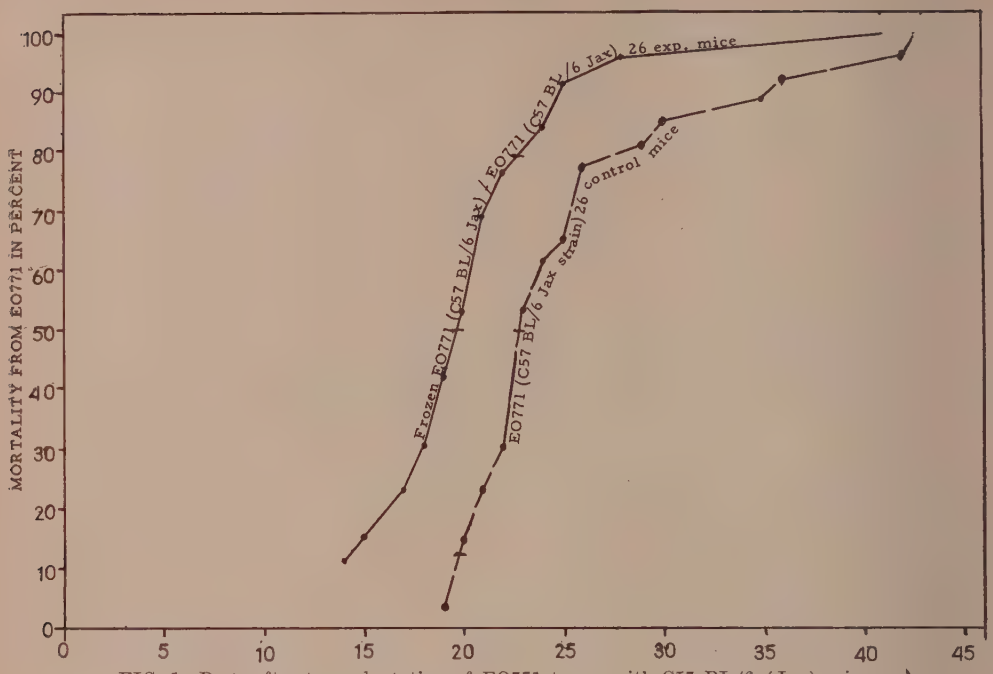

FIG. 1. Days after transplantation of EO771 tumor with C57 BL/6 (Jax) mice.

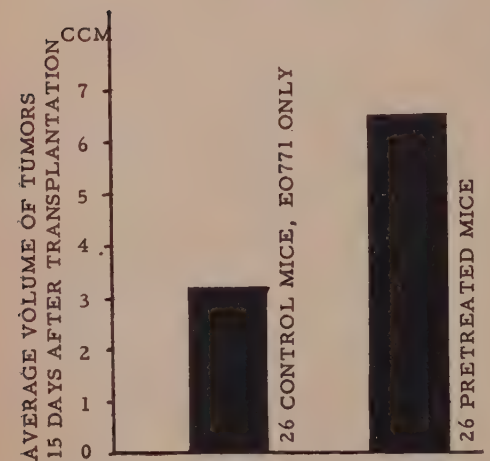

FIG. 2. Growth of EO771 mammary carcinoma in 52 C57 BL/6 mice, 26 of which had been pretreated with non-viable EO771 tumor tissue which had been kept frozen 0°F for 49 days (all 52 mice eventually died of the tumor).

BL/6 mice from the Jackson Memorial Laboratory were inoculated with EO771 mammary carcinoma tissue carried in C57 black/6 mice from the same laboratory. As expected all 52 died from the growth of the neoplasm. However, 26 of the mice which had been pretreated with frozen non-viable EO771 tumor (xyz factor) from the Jackson Laboratory developed a significantly increased rate of tumor growth and earlier mortality than the 26 controls not so treated. The effectiveness for transplanted tumors of the xyz factors in the inbred strain of origin, as demonstrated above, suggests the possibility that spontaneous tumors could be enhanced in the host of origin by xyz factors if such were present in them.

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Received July 3, 1952. P.S.E.B.M., 1952, v80.

A Microbiological Assay for Isonicotinyl Hydrazine. (19709)

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Two essentially different chemical methods have been described for the determination of isonicotinyl hydrazine in biological fluids. The method of Kelly and Poet(1) is dependent on the reaction of the hydrazine portion of the drug with paradimethylaminobenzaldehyde. In the procedure of Rubin *et al.* (2), isonicotinyl hydrazine is decomposed quantitatively into isonicotinic acid and the latter compound is determined by a method employing cyanogen bromide. Since neither chemical assay is specific for the unaltered drug, it was thought that a microbiological method, having a somewhat higher degree of specificity, would be desirable. In the presently described assay, isonicotinic acid is completely inactive and hydrazine has only 1/50 the activity of isonicotinyl hydrazine.

Experimental. Preparation of the assay plates. The test organism employed is a strain of *Mycobacterium butyricum*, obtained from the ATCC in 1943 as their culture No. 362. Stock cultures of the organism are maintained on slants of the following composition:

	g
Glucose	5
Bacto peptone	5
" yeast extract	5
" beef "	5
" agar	17.5
Distilled water to make 1 liter	

The pH is adjusted to 6.8 before sterilization. Abundant growth is obtained after 24 hours incubation at 37°C. Stock slants may be refrigerated for several months without impairing their usefulness. Inoculum for seeding the assay agar is prepared in a medium of the following composition:

	g
Bacto casamino acids	6
Sodium acetate	1
Trigamine‖	.45
dl-tryptophane	.20
Magnesium sulfate hepta hydrate	.10
Dipotassium phosphate	.10
Calcium nitrate	.08
Ferric chloride, hexahydrate	1.25 mg
Copper chloride dihydrate	1 mg
Manganese chloride hexahydrate	.05 mg
Zinc chloride	.05 mg
Glucose (added aseptically after sterilization)	10 g
Distilled water to make 900 ml	

The pH of the medium is adjusted to 6.8 before sterilization and 18 ml amounts are portioned into 125 ml Erlenmeyer flasks. Sterilization is effected at 120°C for 15 minutes. When cool, 2 ml of sterile 10% glucose solution are then added to each flask. For inoculation of the liquid medium a liberal quantity (about one-fourth of a 3 mm loop full) of growth is removed from a stock slant and suspended evenly in the medium. The inoculum is ready for use after 18-24 hours of incubation at 37°C.

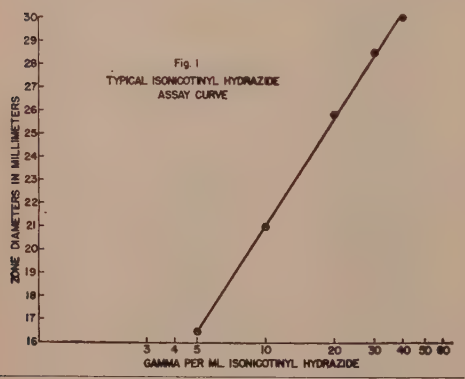
The agar used for pouring the assay plates has the same composition as the liquid inoculum medium except for the inclusion of 1.5% agar. Five ml of this medium are poured into Corning pressed flat-bottom culture plates No. 3162 and allowed to harden. Four ml of the same medium, seeded with 3% v/v of 18-24 hour inoculum, are then carefully and evenly layered over the cooled 5 ml of un-

* Contribution 318.

‖ Obtained from Glyco Products Co., Brooklyn, N. Y.

seeded base agar. For these operations the plates are maintained in a perfectly level position and the agar is held at 48-52° for pouring. When the seed layer has hardened, the assay cups,† previously sterilized, are placed on the agar surface.‡ Gentle heating of the cups to permit sealing to the agar will be found advantageous in the handling of the plates(3). All cups must be heated evenly and uniformly, however, otherwise irregularity of size or distortion of inhibition zones may result. We have found the technic of Schmidt and Moyer(4) convenient and employ 5 cups per plate, using 2 cups for a standard solution containing 20 γ per ml and 3 cups for the sample. The use of 5 replicate plates permits an assay reproducibility of about ± 15% and higher precision can be obtained by the use of a larger number of replicate plates.

Preparation of the sample. To 10 ml of urine in a suitable sized test tube is added 1 ml of concentrated hydrochloric acid. After thorough mixing, the tube is covered to reduce evaporation and held in a boiling water bath for 20 minutes. The sample is then cooled, adjusted to pH 6.5-7.5, and is then diluted to contain from 10 to 30 γ per ml of drug. Equal portions of the sample are then placed in the cups for assay and the plates are incubated at 37°C. The inhibition zone diameters may be read after 18-24 hours.



† Steatite cylinders, 1 mm wall thickness, 8 mm o.d. x 10 mm long, manufactured by Steatite Corp. of America, Keasby, N. J.

‡ When the quantity of the sample is limited, paper discs (Schleicher & Schull No. 740-E) may be used in place of the porcelain cups. The assay curve must then also be set up with paper discs to maintain proportionality.

TABLE I. Analytical Recovery of Known Additions of Isonicotinyl Hydrazine.

Sample	% recovery from urine after addition of 30 γ	100 γ
1	—	93
2	124	100
3	97	97
4	92	100
5	87	91
6	87	102
7	77	97
8	100	98
9	72	88

TABLE II. Comparison of Isonicotinyl Hydrazine Values Obtained by Chemical and Microbiological Assay of Urines.

Sample	Oral dose, mg	Mg isonicotinyl hydrazine excreted as determined by: Microbio assay	Chemical(5) assay
1	125	42	61
2	125	68	65
3	125	51	65
4	190	48	58
5	190	63	79
6	125	50	59
7	190	72	58
8	190	22	48
9	125	29	63

Calculation of results. The assay values are calculated from a standard curve set up according to the method of Schmidt and Moyer (4) with each day's samples. A typical dose-response curve for the concentration range of 5-40 γ per ml is shown in Fig. 1.

Discussion. The method described herein has been found useful in assaying isonicotinyl hydrazine in urines from human and animal subjects submitting to drug excretion studies. As seen from Table I, most samples studied permit good analytical recoveries of known increments of isonicotinyl hydrazine. Moreover, as shown in Table II, fair agreement is obtained with the cyanogen bromide method (2) in the assay of urines from normal subjects.§ These data were obtained on typical

§ We are indebted to Mr. E. DeRitter and to Mr. L. Drekter for access to their data, obtained by the cyanogen bromide assay.

TABLE III. Effect of Hot Acid Treatment on Microbiological Assay of Urines.

Sample	γ/ml isonicotinyl hydrazine found	
	Before treatment	After treatment
1	48	45
2	13	53
3	31	44
4	5	40
5	10	21
6	6	28
7	11	61
8	19	17
9	25	19

24-hour pooled urine specimens, contributed by 9 normal human subjects who had taken orally 125 mg or 195 mg of isonicotinyl hydrazine. The acid-heat treatment is required to release the maximum anti-microbial activity from most urine specimens studied. Occasionally samples are encountered which do not require the acid-heat treatment, such as sample 1, and others are encountered (samples 8, 9) which, even after treatment, do not release all the anti-microbial activity indicated to be present by chemical assay. These data may be seen from Table III. The microbiological activity of aqueous solutions of isonicotinyl hydrazine is not altered by the acid-heat treatment.

Hydrazine has less than 2% the activity of isonicotinyl hydrazine in this assay and isonicotinic acid and isonicotinamide are completely inactive. Combinations of hydrazine and isonicotinic acid have no more activity than that of hydrazine alone. Drug-free urines, when treated as in the isonicotinyl hydrazine assay, have also been found to be completely inactive. Interference will be observed, however, if the urines contain streptomycin, dihydrostreptomycin, aureomycin, terramycin or chloromycetin. Paraamino salicylic acid causes no interference at a concentration of 1000 γ per ml and penicillin is without effect.

Further studies to increase the usefulness of the method are in progress.

Summary. A simple microbiological diffusion-plate assay for isonicotinyl hydrazine has been devised employing *Mycobacterium butyricum* ATCC No. 362 as the test organism. The method is suitable for assaying urines and other aqueous solutions containing 5 γ per ml or more of the drug. The solutions need not be sterile. The assay may be read in 18-24 hours and results are reproducible to ± 15% or closer. Results obtained by this method are in fair agreement with those obtained by the cyanogen bromide chemical assay.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Stimulation of Plant Growth by Antibiotics. (19710)

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Preliminary experiments indicate that under appropriate conditions antibiotics can stimulate the growth of plant tissues, plant organs and whole plants. Although the extent of this phenomenon is at present unknown, tests are under way to determine which plants are affected and which antibiotics are effective. The present communication presents evidence for plant growth stimulation by antibiotics from 3 types of experiments.

Materials and methods. Tissue culture. The material used in these *in vitro* experiments was the virus tumor tissue from the root of the sorrel plant (*Rumex acetosa*).

The basal synthetic medium and the methods used in this type of experiment were the same as those previously described(1-3). Experiments were run in replicates of 5 and an average taken. *Seed germination.* The methods used for testing the effects of antibiotics on the growth of germinating seeds were similar to that described by Allen and Skoog(4). Seeds of the desired varieties were germinated on moistened filter paper in covered Petri plates containing 6 ml of water per plate. The desired concentration of the compound to be tested was included in aqueous solution; control plates contained only distilled water. The number of seeds per plate depended upon the size of the seed: with large seeds such as corn 5 seeds per plate were used, with smaller seeds such as sorrel 10 seeds were used. Germination took place at room temperature with the plates covered to exclude most of the light. Measurements were made at daily intervals after the emergence of the primary root. *Soil studies.* Greenhouse studies on seed germination and subsequent growth in soil were carried out in flats. Control flats were watered with tap water as the dryness indicated. Experimental flats were handled in exactly the same manner except that 5 ppm of the experimental antibiotic were included in the water.

Earlier experiments conducted in 1948 indicated that certain antibiotics in low concentrations stimulated the growth *in vitro* of virus tumor tissue from *Rumex acetosa*(5). The sterile technics employed eliminate the possibility that the stimulation observed is due to the effect of contaminating microorganisms. More recently it has been shown that to maintain satisfactory growth of this tissue vit. B_1 must be added to the medium used in subculturing(6). Its absence will result in death of the tissue after the second transfer.

Results and discussion. The addition of certain antibiotics to the basal medium at a concentration of 1 to 5 ppm in the absence of vit. B_1 will allow apparently normal growth of the virus tumor tissue. The results of this type of experiment are shown in Table I.

Germinating seeds of *Agave toumeyana* were stimulated in their growth by certain concentrations of thiolutin. The maximum effect was found to be at 1.0 to 5.0 ppm. The seeds germinated in the presence of 25 ppm of thiolutin had shorter roots than those treated with the solution containing 5 ppm, however, the coleoptiles were approximately the same length. In this experiment with thiolutin the concentrations ranged from 0.005 to 25 ppm; both sterilized and non-sterilized seeds were used with the same results.

TABLE I. Effect of Various Antibiotics on Growth of Virus Tumor Tissue from *Rumex acetosa.**

Antibiotic added	Concentration	Growth value
None (control)	—	1.3
Penicillin	10 u/ml	4
Terramycin	5 ppm	7.2
Streptomycin	5 "	5.3
Thiolutin	5 "	6.3
Bacitracin	5 "	10.9

* Experimental transfers grown in the absence of all vitamins for 2 weeks prior to experiment. Results are expressed in terms of growth value which is a number determined by dividing the final wet weight of the tissue by its initial wet weight.

TABLE II. Heights of Plants from Control Soil and Soil Treated with 5 ppm of Terramycin.

	Height in inches		
	High	Low	Avg
Sorrel			
Control	8.75	2.25	5.6
Treated	10.13	1.75	9.4
Sweet corn			
Control	19.5	6	14
Treated	24	9.75	17.45

The effects of terramycin on the germination and subsequent growth of sorrel seeds were tested in soil. It was observed that a greater percentage of seeds appeared above ground earlier in the treated flats than in the controls. This difference became more marked in their subsequent development as the plants approached maturity. The seedlings in the terramycin treated flats were definitely larger and more vigorous than those in the untreated flats. In order to determine if there would be a difference after the plants had become larger, these plants were allowed to remain in the flats with no further treatment for 46 days. At this time there were an equal number of plants in both the treated and control flats. The treated plants had an average of

5.2 leaves per plant whereas the control plants had 4.6 leaves per plant. The height of the plants from the ground level was measured. Table II gives the height of the tallest and shortest plants in each group, as well as the average height of all plants in each group in inches. The total weight of plant tissue above ground just as removed from the control flat was 60.9 g and from the treated flat 81.7 g. It is obvious from the foregoing results that the stimulatory effects of terramycin upon the germination and subsequent growth of sorrel plants caused a difference which was still apparent after 46 days.

A group of 49 sweet corn seeds was planted in each of 2 greenhouse flats: one control, one experimental. At the end of 4 weeks the plants were removed from the soil. Of the 49 seeds in the treated flat, 20 had germinated and formed a plant. Of those in the control flat, only 12 had germinated. The height of each plant from ground level was measured. Table II gives the height of the tallest and shortest plant in each group, as well as the average height of all plants in each group in inches. The total weight of plant tissue above ground just as removed from the control flat was 23 g and from the treated flat 45 g. The combined plant tissue from each group was dried at 105°C to a constant weight. The dry weight of all plants in the control group was 2.4 g and in the treated group it was 5.1 g.

Summary. Data from 3 types of experiments have shown a stimulation of plant growth by antibiotics: tissue culture, standard laboratory seed germination, and seed germination and subsequent growth in soil. The use of antibiotics in animal nutrition, particularly in the feeding of swine and poultry, is already prevalent. The preliminary experiments here described suggest the possibility that antibiotics might have a practical application in the stimulation of plant growth as well as in the control of plant diseases(7-9). The use of tissue culture technics should prove a useful tool in the study of the mechanism of stimulatory action by antibiotics.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Intracellular Distribution of Estrogen Inactivating Mechanism in Rat Liver and Other Tissues.* (19711)

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The role of the liver in the inactivation of estrogens has been studied extensively for various species. A comprehensive review of the literature was presented by Paschkis and Rakoff(1). DeMeio and coworkers(2) and Coppedge *et al.*(3) demonstrated the importance of adding disphosphopyridine nucleotide (DNP) and nicotinamide when using male rat liver mince, as contrasted to liver slices, for the inactivation of alpha-estradiol. The object of the study here described was to

* This investigation was supported by research grants from The National Institutes of Health, Public Health Service and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

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localize the enzyme factors concerned with the inactivation of natural and synthetic estrogens through fractionation of rat liver homogenates by differential centrifugation.

Materials and methods. Female rats of the Holtzman-Rolfsmeyer strain, 50 days of age and weighing 130-140 g, were the source of tissues fractionated. Young adult female mice (Carworth Farm CF1 strain), weighing 17-22 g, were used for estrogen assay.

The rats were killed by cervical dislocation and a 10% homogenate of liver or other tissue prepared in 0.25 M sucrose using a cone-shaped glass homogenizer which was cooled in an ice bath. The homogenate was fractionated in the high speed attachment of an International refrigerated centrifuge at a temperature of 3-4°C essentially as described by Schneider(4) except that the nuclei in most cases were washed once only. The fractions obtained with this technic were nuclei, mitochondria, microsomes and supernatant.

Aqueous solutions of the hormones were prepared in phosphate-saline buffer according to Lehninger and Scott(5) except for the omission of glucose. The hormones tested were estradiol-17 β (International hormones), estrone (International hormones), and diethylstilbestrol (The Matheson Co.). The riboflavin-5′-phosphate (monosodium salt) was from Hoffmann-LaRoche, Inc.

The *in vitro* inactivation‡ of the hormones was carried out in 20 cc test tubes as follows: To each tube was added the hormone solution (pH 7.25) containing 3.75 μg of estradiol, 4.7 μg of estrone or 4 μg of diethylstilbestrol, and enough phosphate-saline buffer (pH 7.25) to give a final volume of 6 cc per tube after addition of nicotinamide, DPN and tissue. Just prior to incubation nicotinamide and DPN were introduced to give final concentrations of 0.05 M and 0.0005 M, respectively, and the homogenate or fraction(s) of liver or other tissues tested was added. The quantity of tissue used is expressed as milligrams-equivalent (mg.eq) which denotes the weight of original fresh tissue from which the fraction was obtained. The tubes were incubated for 2 hours at 38°C and then heated for 10 minutes on a boiling water bath to prevent further enzymatic activity.

The estrogenic activity was determined by the uterine weight method using mice ovariectomized 10 to 14 days prior to injection. Four mice were used per dose, each mouse receiving 0.1 cc of the incubation mixture subcutaneously twice daily for 3 days. The incubation mixture was injected without extraction or other treatment. The mice were autopsied 72 hours after the first injection, any fluid expressed from the uteri, and uterine and body weights recorded.

Each incubation mixture had a volume of 6 cc and the quantity injected per assay mouse was 0.6 cc. Thus each mouse received 1/10 of the incubation mixture including the residuum from 0.375 μg estradiol, 0.47 μg estrone or 0.4 μg diethylstilbestrol.

As standard procedure each experiment contained 2 controls. The first consisted of all the components except tissue and was, therefore, a measure of the sensitivity of the assay mice. The second control used each time contained 30 mg.eq. of liver homogenate per tube in addition to the components of the first control. The results obtained with this control measured the estrogen inactivating capacity of the liver of the particular rat whose tissues and tissue fractions were being tested.

In earlier experiments a comparison was made between the first control and one to which previously boiled homogenate was also added. With estradiol, the uterine weights obtained in both were quite comparable. With diethylstilbestrol, however, this is not the case as will be described later. Therefore, the non-tissue control was selected for these experiments.

The uterine weights of both controls varied somewhat from experiment to experiment. The results obtained are given both in absolute (uterine weight) and in relative terms (degree of inactivation).

Results and discussion. The results obtained with estradiol are summarized in Table I, with estrone in Table II and with diethyl-

‡ "Inactivation" as used in this paper refers to loss of biological activity of the hormone as measured by its effect on the uteri of ovariectomized mice.

TABLE I. Inactivation of Estradiol by Homogenates and Fractions of Rat Liver and Other Tissues.

Fraction*	Amt/mouse, mg.eq.†	No. exp.	Mean uterine wt, mg (range)	Relative degree of inactivation, % (range)
Estradiol alone	.375 μg	12	106 (86–133)	0
Hom	3	12	39 (25–57)	100
Hom	6	6	41 (33–51)	94 (78–113)
Hom	15	1	80	38
Boiled Hom	3	2	94 (91–96)	2 (-11–15)
N	6	1	102	9
N	12	2	78 (67–85)	12 (7–16)
Mt	12	2	88 (85–91)	25 (-3–61)
Mt	20	2	88 (83–94)	10 (6–15)
Mc	12	8	88 (65–114)	27 (-3–48)
S	8	2	74 (62–85)	18 (15–21)
S	12,14	2	86 (75–98)	40 (36–43)
Mt 6 and S	6	1	89	–8
Mt 12 " S	8	2	83 (63–103)	37 (31–43)
Mc 6 " S	6	5	52 (31–78)	93 (66–114)
Mc 12 " S	8	9	33 (17–55)	109 (93–122)
Mc 12 " RMP	4.5 μg	3	49 (36–56)	86 (81–91)
Mc 12 " RMP	9 μg	1	41	109
RMP	9 μg	2	74 (71–76)	12 (-5–28)
K–Hom	3	1	103	–25
K–Mc 12 and K–S	8	1	90	–4
K–Mc 12 " L–S	8	1	91	–5
L–Mc 12 " K–S	8	1	18	112
SG–Hom	3	1	81	23
SG–Mc 12 and SG–S	8	1	84	20
SG–Mc 12 " L–S	8	1	76	30
L–Mc 12 " SG–S	8	1	37	85

* Hom = homogenate; N = nuclei; Mt = mitochondria; Mc = microsomes; S = supernatant; RMP = riboflavin monophosphate; K = kidney; SG = salivary glands; L = liver.
† mg.eq. denotes the wt of original fresh tissue from which the fraction was obtained.

TABLE II. Inactivation of Estrone by Homogenates and Fractions of Rat Liver.

Fraction*	Amt/mouse, mg.eq.†	No. exp.	Mean uterine wt, mg (range)	Relative degree of inactivation, % (range)
Estrone alone	.47 μg	4	102 (74–112)	0
Hom	3	4	40 (22–50)	100
Hom	6	2	46 (39–52)	95 (93–96)
N	12	2	73 (71–75)	2 (-2–5)
Mt	20	2	102 (88–117)	13 (-13–39)
Mc	6	1	103	16
Mc	20	2	95 (89–101)	26 (18–34)
S	12,20	2	102 (101–102)	15 (13–17)
Mt 12 and S	12	2	96 (89–103)	24 (9–39)
Mc 6 " S	6	2	52 (42–61)	97 (81–113)
Mc 12 " S	12	3	37 (25–49)	108 (95–125)

* Hom = homogenate; N = nuclei; Mt = mitochondria; Mc = microsomes; S = supernatant.
† mg.eq. denotes the wt of original fresh tissue from which the fraction was obtained.

stilbestrol in Table III. All results are expressed in terms of hormone and tissue administered per mouse, but the amounts incubated *in vitro* in each case were 10 times this quantity, as indicated above.

The relative degree of inactivation was computed separately for each experiment. The mean uterine weight obtained with the incubation mixture containing estrogen but no tissue was designated as 0% inactivation. The mean uterine weight obtained on incubating the estrogen with 3 mg.eq. of liver homogenate was arbitrarily designated as 100% inactivation. All other uterine weights obtained in a

TABLE III. Inactivation of Diethylstilbestrol by Homogenates and Fractions of Rat Liver and Other Tissues.

Fraction*	Amt/mouse, mg.eq.†	No. exp.	Mean uterine wt, mg (range)	Relative degree of inactivation, % (range)
Dieth. alone	.4 μg	10	85 (63–114)	0
Hom	3	10	22 (17–28)	100
Hom	6	1	22	103
Hom	15	1	19	106
N	6	1	48	41
Mt	6	1	52	32
Mt	12	3	70 (62–90)	26 (13–39)
Mc	6	2	57 (52–62)	42 (32–52)
Mc	12	4	41 (27–68)	76 (51–93)
S	3	3	35 (32–38)	83 (77–88)
S	6	5	27 (24–34)	92 (67–99)
S	12	3	25 (20–30)	98 (93–106)
Mc 6 and S	6	4	25 (20–27)	100 (97–107)
Mc 12 " S	12	3	26 (24–28)	97 (95–100)
RMP	4.5 μg	2	21 (19–24)	104 (96–110)
RMP	9 μg	5	24 (20–28)	96 (86–107)
K–Hom	3	1	92	13
K–Mc	6	1	109	–8
K–S	6	1	104	–2
Boiled L–Hom	6	5	25 (19–39)	86 (62–98)
" L–N	6	1	33	69
" L–Mc	6	1	48	40
" L–S	6	2	74 (74–74)	–20 (–30––9)

* Hom = homogenate; N = nuclei; Mt = mitochondria; Mc = microsomes; S = supernatant; RMP = riboflavin monophosphate; K = kidney; L = liver.

† mg.eq. denotes the wt of original fresh tissue from which the fraction was obtained.

given experiment were expressed in terms of the control figures for that experiment. Most of these weights ranged between the uterine weight representing 0 and that representing 100% inactivation. Values less than zero mean that the uteri showed greater stimulation than was produced with estrogen alone. Values above 100 indicate that the inactivation was greater than that obtained when the estrogen was incubated with 3 mg.eq. of homogenate.

The formula used for each experiment to calculate the degree of inactivation is $\frac{C_1 - X}{C_1 - C_2}$ where C_1 is the mean uterine weight obtained with hormone alone, C_2 the mean uterine weight with 3 mg.eq. of liver homogenate, and X the mean uterine weight of each of the other experimental groups tested against the above controls. One of the experiments included in Table I is listed below with its controls to illustrate the method used (the abbreviations are explained at the bottom of Table I):

Fraction	Amt/mouse, mg.eq.	Mean uterine wt, mg	Relative degree of inactivation, %
Estradiol alone (C_1)	.375 μg	89	0
Hom. (C_2)	3	28	100
Mc	12	81	13
Mt	12	91	–3
Mc 12 and S	8	23	108
Mt 12 and S	8	63	43
Mc 12 and RMP	4.5 μg	36	87

Maximum inactivation of estradiol and estrone by liver homogenate occurred when 3 mg.eq. was used. At this concentration of tissue some activity remained as indicated by a uterine weight of 40 mg compared with 24 mg for ovariectomized controls. Six mg.eq.

of homogenate caused slightly less inactivation and in one experiment with estradiol 15 mg.eq. of homogenate gave only 38% as much inactivation as 3 mg.eq. This is in agreement with the finding of Segaloff(6) that with increasing amounts of liver and a standard amount of estradiol increasing instead of decreasing amounts of recoverable estrogenic activity are obtained. This inverse relationship did not obtain with the liver fractions since values above 100% were obtained in some instances with 12 mg.eq. The inactivation of diethylstilbestrol showed no decrease whether 3, 6, or 15 mg.eq. of homogenate was used.

Three mg.eq. of liver homogenate reduced the biological activity of 0.375 μg of estradiol as shown by the reduction in uterine weights from 106 mg to 39 mg (Table I). None of the 4 liver fractions (nuclei, mitochondria, microsomes and supernatant) produced a comparable reduction in activity when used alone in concentrations up to 20 mg.eq. On recombining various fractions it was found that microsomes and supernatant gave inactivation equivalent to that produced by homogenate. With a combination of 6 mg.eq. of each, the inactivation of estradiol was 93% of that obtained with homogenate, and 12 mg.eq. of microsomes and 8 of supernatant gave 109% inactivation.

The factor in the microsome fraction was found to be heat-labile, that in the supernatant relatively heat-stable(7). This suggests that the enzyme(s) is associated with the microsomes and the cofactor(s) with the supernatant.

The supernatant can be replaced by riboflavin monophosphate (RMP). With 12 mg.eq. of microsomes, 4.5 μg of RMP caused 86% inactivation of estradiol and 9 μg of RMP 109% inactivation. This finding may be of interest in view of the deleterious effect of dietary deficiencies on the *in vivo* estrogen inactivating mechanism, reported variously as caused by vit. B deficiency(8,9) or inanition(10,11). Further work on this aspect is needed.

Rat kidney and salivary gland were fractionated and tested to determine whether the inactivating capacity was specific for liver fractions. Neither the homogenate nor the combined microsome-supernatant fractions of kidney or salivary gland inactivated estradiol significantly. When *liver supernatant* was added to the microsomes of either kidney or salivary gland, there was also no significant reduction in activity. However, the addition of kidney or salivary gland supernatant to *liver microsomes* gave results comparable to those obtained with the 2 liver fractions. This indicates that the estradiol-inactivating enzyme(s) found in the liver microsomes is absent in the comparable fraction of the other tissues tested. The necessary cofactor(s), however, seems to be more generally distributed since the supernatant of kidney or salivary gland could be substituted for liver supernatant. The properties of the supernatant are being investigated further at the present time.

Table II summarizes the results obtained when various liver fractions were incubated with estrone. As with estradiol none of the 4 fractions was as effective as homogenate in reducing the biological activity of estrone. When the microsomes and supernatant were combined, 97% inactivation occurred with 6 mg.eq. and 108% with 12 mg.eq.

In most of the experiments with estradiol and estrone the nuclear and microsome fractions were washed only once. In some of these the inactivation obtained was appreciable although still well below that produced by the combined microsome-supernatant fractions. A comparison was therefore made of the various fractions before and after one and two washings. In each case the washings removed most of the inactivating capacity and the washed microsomes were still as effective as the unwashed fraction when combined with the supernatant.

The ability of homogenate and various fractions to inactivate diethylstilbestrol is summarized in Table III. Three mg.eq. of liver homogenate completely inactivated 0.4 μg diethylstilbestrol. This quantity of homogenate was more effective with diethylstilbestrol than with the natural estrogens. A possible explanation is that the conversion products of estradiol and estrone retain some estrogenic activity following incubation with

liver(12), whereas those of diethylstilbestrol appear to be inactive(13).

Liver supernatant inactivated 83%, 92% and 98% of the diethylstilbestrol at concentrations of 3, 6 and 12 mg.eq., respectively. The addition of microsomes had almost no effect in contrast to the results obtained with the natural estrogens.

The value of 76% obtained with 12 mg.eq. of microsomes was possibly due in part to incomplete removal of supernatant from the submicroscopic particles inasmuch as this fraction had not been washed in these particular experiments. A non-enzymatic factor may also be involved and will be discussed later.

When kidney tissue was incubated with diethylstilbestrol, none of the fractions tested inactivated the hormone. Thus in the inactivation of diethylstilbestrol the supernatant fraction is the only one which is necessary and apparently must be from the liver. However, with estradiol the microsomes must be from the liver but the supernatant can be from other tissues. Other differences between the inactivating mechanisms for these 2 estrogens are being studied further, including requirement for DPN, heat lability, etc.

Four and one-half or 9 μg of RMP inactivated diethylstilbestrol in the absence of any tissue. Thus RMP is able to replace liver supernatant in inactivating estradiol and diethylstilbestrol, in the latter case acting alone (a non-enzymatic reaction), in the former in conjunction with microsomes.

Incubation of *boiled* liver homogenate with diethylstilbestrol resulted in the loss of 86% of the estrogenic activity, which indicates that a non-enzymatic mechanism in the liver can also inactivate diethylstilbestrol. Boiled nuclei and boiled microsomes destroyed 69% and 40% respectively of the activity. It is quite possible, therefore, that the relatively high values reported in Table III for the unboiled nuclear and microsome fractions are due mainly to a non-enzymatic mechanism. The inactivation of diethylstilbestrol by supernatant appears to be enzymatic, however, since this fraction after being boiled completely loses its effectiveness.

Summary. Rat liver homogenate was separated into nuclei, mitochondria, microsomes and supernatant fractions by differential centrifugation. The homogenate, the fractions and combinations of fractions were studied for their ability to decrease the activity of estradiol, estrone or diethylstilbestrol when incubated at 38°C for 2 hours in the presence of DPN and nicotinamide. The uteri of ovariectomized mice were used to determine the amount of biological activity remaining.

No single fraction decreased the activity of estradiol markedly. The microsomes and supernatant, when recombined, were comparable to homogenate in inactivating this hormone.

Though the homogenate of kidney or salivary glands was inert in this respect, the supernatant of either tissue when combined with liver microsomes effectively inactivated estradiol. The supernatant could also be replaced by riboflavin monophosphate (RMP). The microsomes of kidney or of salivary glands when used with liver supernatant failed to inactivate estradiol. The inactivation of estrone occurred with the same liver fractions necessary for estradiol: microsomes and supernatant. No appreciable inactivation occurred with any single fraction. For the *in vitro* inactivation of diethylstilbestrol, liver supernatant alone was almost as effective as homogenate. Kidney supernatant was ineffective. RMP alone, in the absence of any tissue, destroyed the estrogenic activity of diethylstilbestrol. A non-enzymatic inactivation of this hormone by liver tissue was also indicated.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Effect of Cystine and Methionine on Healing of Experimental Wounds.* (19712)

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In previous reports(1,2), the more rapid rate of healing of experimental wounds in animals fed a high protein diet as compared to those on a lower protein diet was shown to be due to the greater intake and retention of protein sulfur. It was also shown that methionine could serve as the source of protein sulfur. This explained earlier work which had indicated that methionine increased the rate of healing(3-5). Other essential amino acids (6,7) were found to have no effect on the *healing index,* a numerical measure of a function which is proportional to the rate of healing.

Methionine may have 2 possible roles, involving its sulfur atom, which might affect the *healing index.* First, it might be required directly in the healing processes for such reactions as protein synthesis. Secondly, methionine might serve as a precursor for some other sulfur-containing compound required during healing. Although these alternatives need not necessarily be mutually exclusive, it is not unreasonable to presume that one function will be more important than the other.

In the present paper, it is shown that the *healing index* can be affected by cystine to the same extent as by an equivalent amount of methionine, on the basis of sulfur.

Experimental. The experiments to be described were carried out in a similar manner to those previously reported(1,2). In each experiment, 3 groups of 24 female albino rats (200 ± 20 g) were maintained on a basal diet for 5 days prior to wounding. The basal diet consisted of 6 g casein, 10 g lard, 2 g corn oil, 5 g salt mixture(8), 77 g sucrose, 1500 I.U. vit. A,† 210 I.U. vit. D,† 1 mg thiamine HCl, 1 mg riboflavin, 1 mg pyridoxine HCl, 15 mg nicotinic acid amide, 4 mg calcium pantothenate, 0.5 mg 2-methyl naphthoquinone, 5 mg inositol, and 25 mg choline chloride. This diet would permit only a small amount of protein accretion in normal unwounded animals (as measured by nitrogen excretion and increase in body weight). On the day of wounding, the animals were transferred to the experimental diets. All the animals were given the same weighed amount of diet daily, in quantities which would be completely consumed. Distilled water was permitted *ad libitum.*

Standard experimental wounds were made on the back of the neck of the rats as previously described(1). At approximately weekly intervals, 1/3 of the animals in each group were sacrificed and the tensile strength of a number of 0.5 cm sections of the healing wound were measured(1,9). The relationship of tensile strength to time results in a curve which may be considered to be essentially a straight line. This line can be represented by the equation $T = kt + C$, where T is the tensile strength in grams, and t, the time in days. C is a constant. The slope of this line (K) is the *healing index,* and may be computed from the equation:

$$K = \frac{T_2 - T_1}{t_2 - t_1}$$

where T_1 is the tensile strength at time t_1, and T_2 is the tensile strength at time t_2. K

* This work was done under contract with the U. S. Navy, Office of Naval Research.

† From oleum percomorphum.

TABLE I. Effect of Experimental Wounds on the Sulfur and Nitrogen Balances. Exp. I.

Group No.	Casein in diet, %	Amino acid supplement	Nitrogen per 100 g diet, mg	Sulfur per 100 g diet, mg	Nitrogen: Avg daily intake, mg	Nitrogen: Avg daily retention, mg	Sulfur: Avg daily intake, mg	Sulfur: Avg daily retention, mg	"Excess sulfur," mg	*Healing index*
I	6	Alanine	910	48	64.8	6.9	3.4	1.53	1.07	40
II	6	Methionine	910	248	64.8	22.1	17.7	4.15	2.68	50
III	6	Cystine	910	248	64.8	22.7	17.7	4.22	2.64	51

has the dimension of a rate term which describes a function of the rate of healing.

Urine samples were collected daily before and after the time of wounding. The urine was stored under toluene at 5°C until analyses were run. The urine was analyzed for total nitrogen (microkjeldahl) and sulfur(10,11).

Results and discussion. The nitrogen and sulfur sources of the diet fed the wounded animals used in Exp. 1 are described in Table I. The *healing indices* for these animals were calculated from the tensile strength data plotted in Fig. 1. It can be seen that the rates of healing in the animals receiving the methionine and cystine supplements ($K = 50$ and 51) are significantly greater than that of the control group ($K = 40$). It should be noted particularly that equivalent amounts of cystine and methionine (on the basis of sulfur) have the same effect on the *healing index*. A repetition of this experiment gave identical results.

A correlation between the sulfur retention and the *healing index* was observed here, as in previously reported work(1,2). It would be expected that the retention of sulfur should be proportional to the retention of nitrogen, in a ratio similar to that found in the animal. In the rat, the nitrogen:sulfur ratio is approximately 15:1. After wounding, there appears to be a greater retention of sulfur than might be expected from the amount of nitrogen which is retained. This "excess sulfur" also appears to be correlated with the *healing index*. The data supporting this correlation are shown in Table I.

Whether the results noted above were due to the direct action of the cystine supplement, or to the effect of the cystine in sparing the methionine available from the casein in the diet, remained to be determined. Therefore, an experiment similar to the previous one was carried out, except that the casein was omitted from the diet fed the wounded animals. The control group of animals received a diet containing no sulfur amino acids and 44 mg of amino acid nitrogen per 100 g diet, in the form of alanine. The cystine and methionine supplemented diets contained 100 mg of amino acid sulfur and 44 mg of nitrogen per 100 g of diet. The curves of tensile strength against time obtained in this experiment are plotted in Fig. 2.

Here again, the effect of cystine and methionine on the *healing index* can be seen to be essentially the same ($K = 36$ and 34) and significantly greater than that found in the

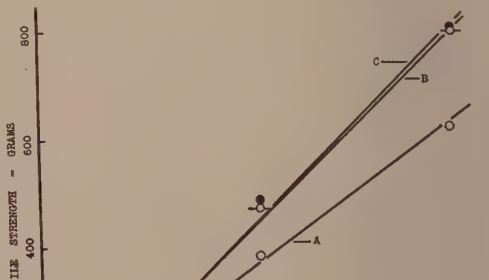

FIG. 1. Tensile strength of healing wounds in rats on a 6% casein diet plotted against time. Curve A (Group I), alanine supplement, *healing index (K)* = 40; Curve B (Group II), methionine supplement, *(K)* = 50; Curve C (Group III), cystine supplement, *(K)* = 51. The significance between mean values of tensile strength for Groups I and II is "p" = <.01.

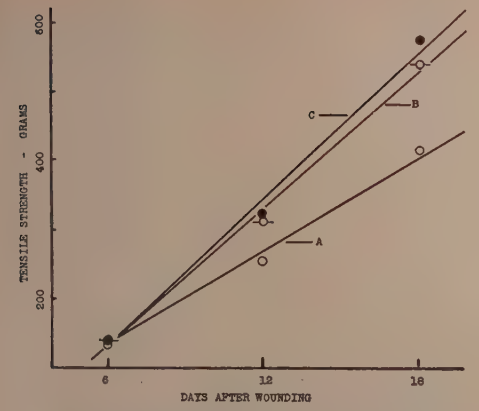

FIG. 2. Tensile strength of healing wounds in rats on a non-protein diet plotted against time. Curve A (Group I), alanine supplement, *healing index (K)* = 24; Curve B (Group II), methionine supplement, $K = 34$; Curve C (Group III), cystine supplement, $K = 36$. Significance between mean values of tensile strength for Groups I and II is "p" = <.01.

controls ($K = 24$). Since the conversion of methionine to cystine is irreversible *in vivo* (12,13), it must be concluded that the methionine in the diet is first converted to cystine before it becomes available for the healing processes. It then appears that cystine is the limiting factor affecting the *healing index,* and that methionine serves primarily as a source of cystine sulfur. Of course, cystine, as such, may not be required by the healing wound. It may be that cystine is itself merely a precursor of the sulfur containing substance utilized during healing. The correlations between the *healing indices,* the sulfur balances and the "excess sulfur" values for this experiment are shown in Table II.

Further work has indicated that methionine, *per se,* is required to some extent during wound healing, over and above that which may be converted to cystine. When rats were fed a 5% casein diet, supplemented with 100 mg of ethionine sulfur and 100 mg of either cystine or methionine sulfur per 100 g diet, a lower *healing index* was observed than in the control animals, who received no sulfur amino acid supplement. A comparison of the tensile strength data for this experiment is shown in Fig. 3. These data may be interpreted to mean that the ethionine is interfering with the utilization of both the cystine and the methionine.

Ethionine is known to block the conversion of methionine to cystine as well as the incorporation of methionine into protein(14,15). The latter effect results in a decreased rate of protein synthesis. In Group II (methionine supplement), the low *healing index* may be considered to be due to the lack of cystine resulting from the interference with methionine conversion. However, in spite of the relatively large amounts of cystine available to the animals in Group III, a low *healing index* was still observed. In this case, it seems probable that the ethionine interfered with the utilization of methionine for purposes other than cystine formation, so that the cystine requirement was no longer the limiting factor in the healing process. It must then be concluded that methionine is also required for wound healing. It is not unlikely that the methionine requirement during wound healing is needed primarily for protein synthesis, whereas, the cystine required may be used, to some extent, for reactions other than protein synthesis.

In the experiment where the diet fed to the wounded rats contained no protein, the methionine required for healing by the control animals and by those receiving the cystine supplement must have originated in the tissue protein. It would then be reasonable to think that the methionine requirement must be

TABLE II. Effect of Experimental Wounds on Nitrogen and Sulfur Balances. Exp. II.

Group No.	Nitrogen		Sulfur			
	Avg daily intake, mg	Avg daily retention, mg	Avg daily intake, mg	Avg daily retention, mg	Avg "excess sulfur," mg	*Healing index*
I	3.1	−40.1	0	−2.06	.60	24
II	3.1	−35.9	7	1	1.39	34
III	3.1	−35.6	7	1.04	1.33	36

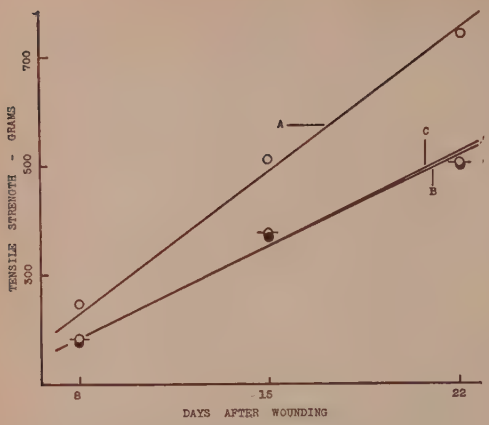

FIG. 3. Tensile strength of healing wounds in rats on a 5% casein diet plotted against time. Curve A (Group I), alanine supplement, *healing index (K)* = 31; Curve B (Group II), ethionine and methionine supplement, $K = 21$; Curve C (Group III), ethionine and cystine supplement, $K = 21$. Significance between mean values of tensile strength for Groups I and II is "p" = .01.

relatively small as compared to the cystine requirement.

Summary. The effect of methionine and cystine on the *healing index* of standard experimental wounds in rats was determined. Since both amino acids have the same effect, per equivalent of sulfur, it is concluded that methionine is converted to cystine before being used in the healing process. When the utilization of methionine is blocked by ethionine, cystine is ineffective, indicating that some methionine, *per se,* is required for the healing of wounds. There appears to be a correlation between the *healing index* and the retention of amino acid sulfur in excess of that expected on the basis of nitrogen retention.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Effects of Auxotrophic Mutations on the Adaptation to Inositol Degradation in *Aerobacter Aerogenes.** (19713)

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Enzymatic adaptation and cell division in microorganisms are closely allied. Both processes are inhibited by the same agents, and in many instances only dividing cells are capable of adaptation. When adaptation can occur in the absence of a source of exogenous nitrogen, lower levels of adaptive enzymes are attained than in media capable of supporting growth(1). It has been postulated that in resting cells an interconversion of enzymes is responsible for adaptation(1). On the other hand, very recently, evidence has been presented that resting yeast cells contain

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a pool of free amino acids and that adaptation can be prevented by amino acid analogues, suggesting synthesis of adaptive enzymes from a reserve of simple nitrogen compounds(2). In addition, the ribonucleic acids of the cell may be involved in adaptation.

The present paper reports preliminary results of an attempt to elucidate some of the phases of the mechanism of enzymatic adaptation with the use of amino acid, purine and pyrimidine-deficient mutants.

Previous studies in this laboratory have shown that the ability of capsulated strains of *Aerobacter aerogenes* to grow on *myo*-inositol as the only source of carbon is due to the formation of adaptive enzymes. Resting cell suspensions of this organism can adapt to the oxidation of inositol in 30-60 minutes(3). The adaptation seems to involve the synthesis of several enzymes, acting on a series of compounds intermediate in the degradation of *myo*-inositol(3,4). The inositol system was used in the present study, in the hope that the results would contribute to the understanding of enzymatic adaptation in general, as well as to the specific problem of inositol metabolism.

Materials and methods. Myo-inositol (formerly called *meso*-inositol or i-inositol) was obtained from the Corn Products Refining Co. *D,L-tryptophane,* and *L-histidine* were Merck preparations. *D,L-leucine* was a product of Hoffmann-LaRoche, and *guanine* and *uracil,* products of the Nutritional Biochemicals Corporation. The purity of these compounds was checked by paper chromatography and ultraviolet spectroscopy.

The salt base used in the growth experiments and as suspending medium in the manometric experiments consisted of Na_2HPO_4 0.54%, KH_2PO_4 1.26%, $MgSO_4 \cdot 7H_2O$ 0.02%, $CaCl_2$ 0.001%, pH 6.5.

Aerobacter aerogenes 1033, a capsulated strain, giving the characteristic biochemical reactions of this group was originally isolated from a patient at Boston City Hospital. It has been used in biochemical studies of inositol and glycerol metabolism in this laboratory(4,5). The *amino acid-deficient mutants* were obtained after ultraviolet irradiation by the penicillin selection method of Davis(6).‡

TABLE I. Growth Requirements of Mutants.

Strain	Requirement	Level for maximal growth on glucose (μg/ml)
44	Tryptophane	20
50	Histidine	20
54	Leucine	90
P-12	Uracil	20
P-14	Guanine	20

The *purine* and *pyrimidine-deficient mutants* were prepared by an analogous procedure: a suspension of cells of strain 1033 was irradiated by an ultraviolet lamp, adjusted to kill 99.9% of the cells. The survivors were cultured in a medium enriched with beef heart infusion at 37°C overnight. Several dilutions of the centrifuged cells were then inoculated into minimal broth containing 1,500 units of penicillin per ml, and incubated at 37°C for 5 hours.

Two series of minimal agar plates containing mixtures of purines and pyrimidines and their ribonucleosides and nucleotides at levels of 8 μg and 0.8 μg per ml were inoculated with 0.1 ml volumes of the penicillin broths. After 48 hours of incubation small colonies were picked and their requirements determined on agar plates and in liquid cultures. Five mutants were isolated in one irradiation experiment.

The minimal level of growth factor required for maximal growth was determined by shaking the cultures consisting of 50 ml of salt base, containing 0.2% glucose, 0.2% ammonium sulfate, and graded amounts of the growth factors, on a Camp type shaker for 16-18 hours at 37°C. The turbidity of the cultures was measured, after appropriate dilution, at 590 mμ in a Coleman spectrophotometer, Model 11. The same maximal turbidity was obtained by mutants and wild strain.

The growth requirements of the mutants and the amount of enrichment needed for maximal growth under these conditions are summarized in Table I. The amino acid-deficient mutants had requirements that could not be replaced by other amino acids; the

‡ We are indebted to Marcus S. Brooke for the preparation of these mutants.

purine and the pyrimidine mutants were less exacting: Strain P-12 which was found to excrete orotic acid, could utilize uracil, cytosine, their nucleosides and nucleotides, and thymine, although growth on the latter was very slow. Strain P-14 could grow on guanine, guanosine and guanylic acid, but not on adenine and its derivatives. All strains were maintained by weekly transfers on agar enriched with tryptic digest (amino acid-deficient mutants) or with beef heart infusion (purine-pyrimidine-deficient mutants).

The rates of growth of the mutant strains on glucose and on *myo*-inositol were determined in cultures consisting of 50 ml of salt base containing 0.2% of the carbon source, 0.2% $(NH_4)_2SO_4$, and the minimal level of growth factor listed in Table I; 3 ml of a similar culture, with glucose as carbon source, shaken for 16-18 hours at 37°C, served as inoculum. The cultures contained in 125 ml Erlenmeyer flasks, were shaken at 37°C; samples were withdrawn at half-hourly intervals and their turbidity measured.

Oxygen uptake was measured in a conventional Warburg apparatus. The cells were grown as described above, on glucose, and with the minimal level of growth factor required for maximal growth. It was found that most consistent results were obtained in the manometric experiments, when 0.5 ml of a 10% solution of glucose was added to 50 ml cultures after overnight growth, and the shaking continued for an additional period of 30 minutes. This treatment apparently restored the metabolic activities of the cells which had suffered from the earlier exhaustion of glucose in the growth medium. All cell suspensions used in the experiments reported here were treated in this manner.

The cells were collected by centrifugation at 5000 r.p.m. in the cold, and washed twice with 0.9% NaCl solution. They were suspended in the salt base containing, when indicated, 0.2% $(NH_4)_2SO_4$, and the growth factor at the levels listed in Table I, or at one tenth of that level. The concentration of the cells was adjusted to an optical density of 1.2 at 590 mμ; 2.5 ml portions of the cell suspension were placed in the main compartment of the Warburg vessels and 20 μM of *myo*-inositol in 0.5 ml of H_2O in the side arm. The center well contained 0.2 ml of 20% KOH.

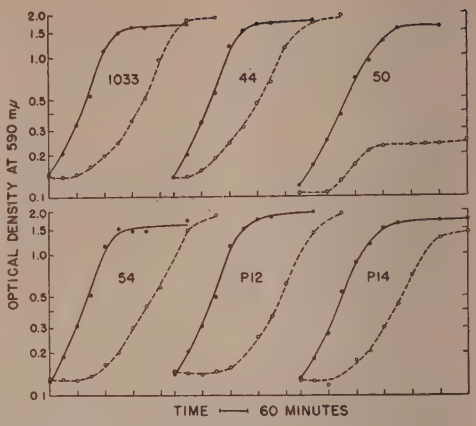

FIG. 1. Growth curves of the wild strain and of the mutants on glucose (full line), and on inositol (dotted line), in minimal medium with the enrichments shown in Table I.

In all the experiments reported here the mutant and the parent strain 1033 were tested simultaneously. All Warburg vessels contained salt base and cells. Vessels of each set varied by containing the following additional materials: 1, none; 2, 0.2% $(NH_4)_2SO_4$; 3, growth factor (0.1 of minimal level); 4, 0.2% $(NH_4)_2SO_4$ + growth factor (0.1 of min. level); 5, growth factor (min. level); 6, 0.2% $(NH_4)_2SO_4$ + growth factor (min. level). The vessels were shaken at 35°C. Readings were taken at 10-minute intervals. The oxygen uptake was measured for 30 minutes, then the *myo*-inositol was tipped in from the side arms, and readings taken for a period of 100 minutes. The cell density was determined at the end of the experiment.

Results and discussion. The growth curves of the parent and the mutant strains on glucose and on *myo*-inositol are shown in Fig. 1. At the level of growth factor required for maximal growth on glucose, the mutants grow on glucose, adapt to *myo*-inositol, and grow on *myo*-inositol at the same rate as the parent strain. The same level of growth was obtained on glucose as on inositol in all except the histidine requiring mutant, strain 50; in order to obtain full growth on inositol it was necessary to add 600 μg which is 30 times as much

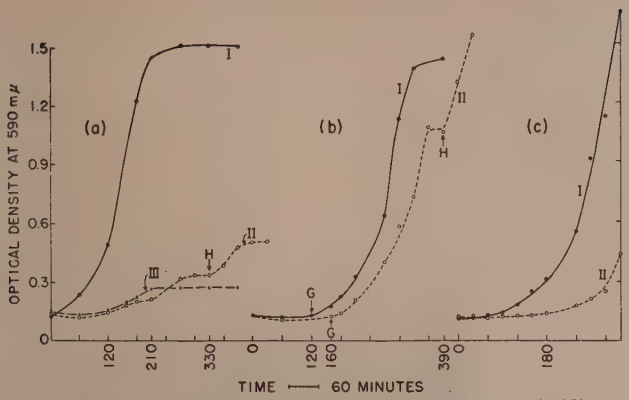

FIG. 2. Effect of histidine on growth of strain 50. a) Utilization of histidine. Curve I—Glucose and 20 μg/ml of histidine; Curve II—Inositol and 20 μg/ml of histidine; Curve III—Inositol and 40 μg/ml of histidine. Arrows mark addition of 20 μg/ml of histidine. b) Degradation of histidine. 20 μg/ml of histidine. Glucose (G), and histidine (H) added where indicated by arrows. c) Growth on 600 μg/ml of histidine. Curve I—with inositol; Curve II—without inositol.

as was necessary for full growth on glucose. This unexpected consequence of the inability of a mutant to synthesize histidine was one of the most striking observations made in the present study. The increased level of histidine was required by adapting as well as by previously adapted cells as shown by the experiment in Fig. 2. In this experiment the addition of histidine to the medium in which growth on inositol had ceased, resulted in an immediate resumption of growth which, however, came to a halt after an increase in cell density similar to that attained in the first period of growth. This cessation of growth was due to the exhaustion of histidine, because addition of fresh histidine resulted again in immediate growth. In the absence of a source of carbon, histidine was not broken down during the first 150 minutes. When glucose was added during this time interval as much growth was obtained as in the case when both histidine and glucose had been present from the beginning (Fig. 2). Exposure of histidine to the cells beyond 150 minutes apparently caused adaptive degradation of histidine. Lower levels of growth were reached when glucose was added after this time interval, and growth occurred with histidine at 600 μg per ml as the only source of carbon (Fig. 2).

The enormous increase in the histidine requirement in cells growing on inositol may be due to the production of inositol degrading enzymes excessively rich in histidine. In that case, inositol grown cells should contain many times as much histidine as glucose grown cells. However, histidine does not seem to be stored to an appreciable extent in either glucose or inositol grown cells, as shown by their inability to grow on glucose in the absence of histidine. Yet, glucose grown cells are able to form adaptive enzyme for inositol degradation in the absence of histidine (Fig. 4). The increased requirement for histidine by cells growing on inositol thus does not seem to be due to a utilization of histidine in large amounts for the production of inositol degrading enzymes, but rather to be connected with some aspect of inositol metabolism. *Myo*-inositol which is degraded by a pathway distinct from that by which glucose is broken down(4), may fail to provide a building block which can be formed from histidine. This interesting relationship between inositol and histidine metabolism will be further investigated.

The results of the manometric experiments are illustrated in Fig. 3 and 4. Fig. 3 compares the kinetics of adaptation of the wild strain and the tryptophane requiring mutant, strain 44, and Fig. 4 summarizes the results obtained with all the strains. The results ex-

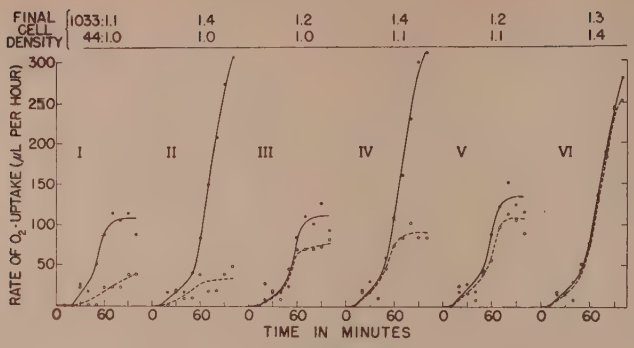

FIG. 3. Rate of oxygen uptake on inositol by strains 1033 (full line) and 44 (dotted line). Additions: I—None, II—$(NH_4)_2SO_4$, III—2 μg/ml tryptophane, IV—$(NH_4)_2SO_4$ + 2 μg/ml tryptophane, V—20 μg/ml tryptophane, VI—$(NH_4)_2SO_4$ + 20 μg/ml tryptophane. Original optical density of the cell suspensions was 1.

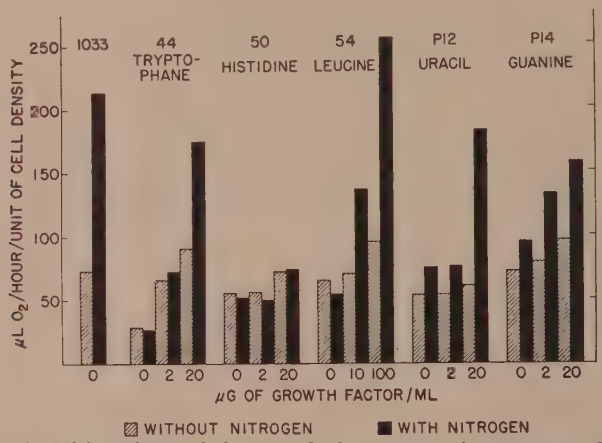

FIG. 4. Effect of addition of growth factor and of exogenous nitrogen on production of adaptive enzyme. Amount of adaptive enzyme formed by 3 ml of a cell suspension with an optical density of 1 is expressed in μL of oxygen taken up in one hr, and was calculated from: $R_{100}/D_{100}—R_o/D_o$, where R stands for rate of oxygen uptake and D for cell density, at the time of addition of inositol and at 100 min.

pected by analogy with other adaptive processes in bacteria and yeasts were observed with the parent strain 1033. Adaptation took place in the absence of added $(NH_4)_2SO_4$, but considerably higher levels of adaptive enzyme were attained in its presence. Under the latter condition an increase in cell density was observed, but was insufficient to account for the increase in the concentration of adaptive enzyme, as shown in Fig. 4. The addition of the growth factors to the wild strain, either in the absence or presence of $(NH_4)_2SO_4$, had no appreciable effect on the adaptation.

The auxotrophic mutants, on the other hand, could achieve similarly high levels of adaptive enzyme in an $(NH_4)_2SO_4$ containing medium only when the individual growth factor was also supplied, that is, the highest levels of adaptive enzyme were attained only by dividing cells. This relation between cell division and adaptation is well illustrated by the behavior of the histidineless mutant, strain 50. The level of histidine supplied was insufficient for growth as discussed previously, and similarly inadequate for the production of large amounts of adaptive enzyme.

A more specific role of some of the growth factors in adaptation could be demonstrated

by experiments in which cell suspensions of the mutants were exposed to inositol in media incapable of supporting growth. It was found (Fig. 3) that the loss of the ability to synthesize tryptophane in strain 44 reduced the amount of adaptive enzyme formed by this strain to about one-third of that formed by the wild strain. The ability of strain 44 to produce adaptive enzyme in the absence of growth could be restored by the addition of tryptophane, but not by the addition of $(NH_4)_2SO_4$.

Other experiments provided evidence that lack of added tryptophane interfered neither with the oxidation of glucose nor, in adapted cells, with the oxidation of inositol; regardless of the absence or presence of tryptophane the oxygen uptake on either glucose or inositol amounted to the theoretical one (6 M/M) in 0.0001 M 2,4-dinitrophenol, but was lower (3 M/M) without dinitrophenol. This latter finding shows that the normally occurring oxidative assimilation, which accounts for the low oxygen uptake(7), does not depend on the presence of the growth factor. The demonstration that assimilation, an energy requiring process, can take place in the absence of added tryptophane, makes it unlikely that the inability of mutant 44 to adapt under these conditions is due to an impairment of the energy metabolism of the cell.

The ability of the leucineless and the histidineless mutant to adapt in the absence of added growth factor was only slightly less than that of the wild strain. Still, addition of leucine and of histidine, respectively, stimulated adaptive enzyme formation, while addition of $(NH_4)_2SO_4$ alone was without effect.

In contrast to these findings are the observations made on the adaptation of the pyrimidineless and the guanineless strains. Ammonium sulfate alone caused a small increase in the production of adaptive enzyme; addition of uracil alone to strain P-12 was without significant effect, and addition of guanine alone to strain P-14 was less effective than addition of ammonium sulfate; the improvement in adaptation caused by the addition of guanine might be explained by the ability of guanine to act as an amino donor. As was observed with the amino acid requiring mutants, the highest levels of adaptive enzyme were attained only when cell division could occur, *i.e.*, in the presence of both ammonium sulfate and growth factor.

The observations on the adaptation of auxotrophs in media not capable of supporting growth may be summarized as follows: while the synthesis of adaptive enzyme is limited by the lack of the specific growth factor in the amino acidless mutants, it is limited by the lack of an exogenous source of nitrogen in the guanine- and the pyrimidineless mutants.

The bearing of these findings on the problem of adaptive enzyme synthesis may now be considered briefly. The stimulation of adaptation by the amino acids required for growth seems to suggest that even in the absence of cell division adaptive enzymes are synthesized *de novo* from amino acids. The ability of the amino acid auxotrophs to form adaptive enzyme, though in smaller amount, in the absence of the required growth factor, may be due either to the storage of the amino acid or perhaps to the liberation of the amino acid from proteins by hydrolysis. Such an indirect utilization of proteins for formation of new enzymes might explain the competitive interactions observed in non-proliferating cells(1).

Uracil and guanine do not seem to be directly involved in the synthesis of adaptive enzyme. The stimulation of adaptation in these auxotrophs by ammonium sulfate in the absence of growth constitutes further evidence for the biosynthesis of new enzyme from simple nitrogen compounds.

The observation that dividing cells can attain a level of adaptive enzyme several times as high as that attained by resting cells (Fig. 4) suggests that still other factors in addition to the inducing agent, the building blocks, and energy are involved in the control of adaptation.

Finally, the preliminary nature of the results reported here must be emphasized: only one substance, *myo*-inositol, has been used to induce adaptation and the responses of only 5 auxotrophs have been recorded. We hope to test the usefulness of this approach and the general validity of the conclusions drawn by extending this study to other un-

related systems and to additional mutants of different types.

Summary. The ability of amino acid and of purine and pyrimidine auxotrophs of *Aerobacter aerogenes* to form adaptive enzymes for the degradation of *myo*-inositol has been investigated. It was found that adaptive enzyme synthesis by the amino acid auxotrophs could be stimulated in the absence of growth by addition of the required nutrilite, but not by addition of $(NH_4)_2SO_4$. In contrast, adaptation in the guanine and pyramidine auxotrophs was enhanced by addition of $(NH_4)_2SO_4$ alone. In all cases, dividing cells attained higher levels of adaptive enzyme than resting cells. The bearing of these results on the mechanism of adaptation is discussed. The poor utilization of histidine by strain 50 for growth on inositol does not seem to be due to the effect of histidine on adaptation, but rather seems to suggest a metabolic relationship between inositol and histidine.

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Received July 8, 1952. P.S.E.B.M., 1952, v80.

Response of X-irradiated Mice to Intravenous Inoculation of Intestinal Bacteria.* (19714)

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The possibility that infection by bacteria of intestinal origin plays a significant role in death following exposure to ionizing radiation has been suggested by a number of observations. Bacteria common to the lower intestinal tract have been found in blood and organs of animals after total body X-irradiation(1-4). Miller *et al.*(5,6) showed a high incidence of bacteremia identified with normal intestinal inhabitants in mice during the second week after exposure to 450 or 600 r total body X-irradiation. This was the period of greatest mortality. Furthermore, it has been possible to reduce mortality among irradiated animals by the administration of various antibiotics(7,8). The protective effect was considerably reduced in mice if there was a prevalence of invading intestinal bacteria which were relatively insensitive to the antibiotics(7). Increased susceptibility of mice to inoculation with *Escherichia coli* following sublethal total body X-irradiation was shown by Shechmeister *et al.*(9,10). Subcutaneous or intraperitoneal injection of *E. coli* caused much higher mortality among irradiated mice than among normal animals receiving the same inoculum.

The present experiments were undertaken to study the effect in mice of intravenous inoculation of another normal intestinal organism, a member of the genus *Proteus*, after moderate total body X-irradiation.

Experimental procedure. C_3H mice, 60-90 days of age, were subjected to total body X-irradiation in groups of 3 in plastic (Styron) containers. The radiation was delivered from a 200 kv Picker-Waite X-ray therapy machine operating at 20 ma and a distance of 20 cm. The filters consisted of 0.5 mm Cu and 1.0 mm Al. Doses ranged from 400 to 550 r, given at a rate of 230 r per minute. Non-irradiated controls were kept in the plastic containers for the same length of time as the irradiated animals. The mice were housed in plastic cages in groups of 6 in an air-condi-

* This work was conducted under Contract No. AT (30-1)-1123 between the U. S. Atomic Energy Commission and Brown University, Providence, R. I.

tioned room at 72°C and were maintained on a commercial stock ration.

Three days after irradiation the mice were inoculated in the tail vein with 2-5 million *Proteus* contained in 0.2 ml saline. Groups of animals given the same X-ray dose followed in 3 days by inoculation with 0.2 ml sterile saline and groups not irradiated but injected with 2-5 million *Proteus* were included as controls. All mice were observed for 30 days after irradiation to obtain mortality data. The bacterial inoculum was prepared by suspending the growth from several 24-hour agar slants in sterile saline, estimating the number of organisms from turbidity-plate count data, and diluting with sterile saline as required. A plate count was made on this suspension to check the number of organisms injected. The culture of *Proteus* used was obtained from heart blood of a mouse given X-irradiation only. When originally isolated, the organism had the biochemical characteristics of *Proteus vulgaris*. After having been maintained as a stock culture for over a year, its properties more closely resemble those of *Proteus morganii*. Sucrose and maltose are usually not fermented, although slow acid production is occasionally observed in these sugars. The organism no longer produces H_2S in lead acetate agar and attacks urea more slowly than when first isolated. Similar changes in apparent *P. vulgaris* cultures have previously been observed after maintenance in stock culture(11). Both *P. vulgaris* and *P. morganii* have been found in cecal content and feces of our mice.

In experiments at 500 and 550 r, tail blood samples were taken from mice in each of the 3 groups to determine the number and kind of bacteria present. Blood was taken within one minute after inoculation and daily thereafter for 15 days. The tail was dipped in tincture of iodine, allowed to dry, snipped with sterile scissors, and a measured amount of blood taken in a sterile red blood cell diluting pipette. Serial dilutions were made in 0.85% sodium chloride solution containing 0.5% sodium citrate. One-half ml amounts of various dilutions were spread over the surface of Levine's eosin-methylene blue agar (Difco) and Bacto heart infusion agar (Difco) plates. A small sample of whole blood taken in a capillary pipette was plated directly on these media. The tail was cauterized after taking the samples. Plate counts were made after 48 hours incubation at 37°C. Cultures for identification procedures were isolated at this time. Mice which were bled were not tabulated in survival data.

TABLE I. Effect of Intravenous Inoculation of *Proteus* on Mortality of X-Irradiated Mice.

X-ray dose	Inoculum	Mortality* Dead/ Total	Mortality* % dead	Median time of death (days post-x-ray)
400r	*Proteus*†	6/14	43	9.5
400r	Saline	0/12	0	—
500r	*Proteus*	20/20	100	6
500r	Saline	3/18	17	12
550r	*Proteus*	12/12	100	7
550r	Saline	5/12	42	16
None	*Proteus*	0/34	0	—

* Mortality data based on observation of mice for 30 days after irradiation.

† No. of *Proteus* inoculated ranged from 2–5 × 10^6.

Results and discussion. More deaths occurred among irradiated mice inoculated with *Proteus* than in control mice given the same irradiation followed by sterile saline injection. At 500 and 550 r the death rate was increased to 100% by *Proteus* inoculation. There were no deaths among non-irradiated animals given the same number of *Proteus*. At all 3 X-ray doses, the median time of death in days after irradiation was shorter for mice inoculated with *Proteus* than for those receiving sterile saline. The results are summarized in Table I. These data show an increased susceptibility to infection with a normal intestinal organism in mice subjected to moderate total body X-irradiation. Such heightened susceptibility to infection among irradiated animals has previously been shown with various pathogenic organisms(12,13) and *E. coli*(9,10). The increased number and earlier occurrence of deaths caused by injection of *Proteus* after moderate doses of X-ray support the concept that infection with intestinal bacteria is important in radiation death. These observations are evidence against the idea that infection is merely a terminal event in death following irradiation.

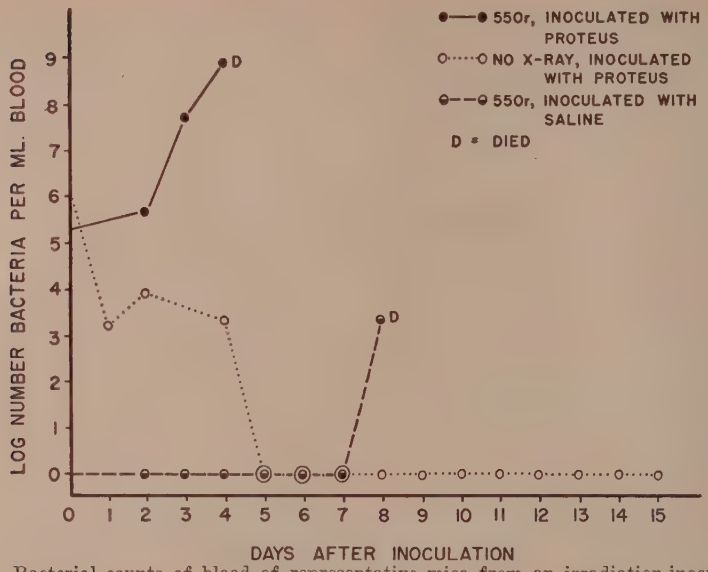

FIG. 1. Bacterial counts of blood of representative mice from an irradiation-inoculation experiment at 550 r.

The plate counts on daily blood samples indicate that the animals subjected to 500 or 550 r had little ability to restrict multiplication of the inoculated bacteria. The organisms increased in number until death occurred, generally rising to 10-100 million per ml blood. Apparently the defense mechanisms related to blood clearing were greatly impaired by irradiation. Chrom(2) observed reduced blood clearing capacity in irradiated mice injected with the Breslau bacillus (probably *Salmonella typhimurium*. The non-irradiated mice were able to remove the inoculated *Proteus* from the blood comparatively well. The number was reduced markedly during the first day after inoculation, but some were found in the blood for several days, usually only a few thousand or less per ml. By the 11th or 12th day after inoculation the blood was free of bacteria. The irradiated, saline-injected mice rarely showed any bacteremia until 7 or more days after inoculation (10 or more days after irradiation). Bacteria found in the blood subsequent to this time were *Proteus* species, unidentified streptococci, and, on one occasion, a *Pseudomonas*. These organisms were presumably invading from the intestine. Both the types of bacteria found and the time of occurrence of bacteremia were similar to the findings of Miller *et al.*(5). At 550 r all mice which died in the group injected with saline and repeatedly bled showed bacteremia before death. However, at 500 r an occasional animal in this group died without showing bacteremia. More deaths occurred at both X-ray doses among mice subjected to daily bleedings in the group given saline than among those merely observed for mortality data. Thus the course of events in the mice from which repeated blood samples were taken may not be strictly comparable to that among those not bled. Fig. 1 shows the results of blood cultures on representative animals from an experiment at 550 r.

Summary. Intravenous inoculation of mice with *Proteus* following moderate total body X-irradiation caused a marked increase in mortality and a much shorter median death time compared to control animals injected with saline. A severe bacteremia existed before death in these animals. Non-irradiated mice given the same inoculum of *Proteus* quickly reduced the number of bacteria in the blood with eventual complete clearing. No deaths occurred among these animals. Bacteremia caused by organisms common to the

intestinal tract frequently occurred in the mice which died following irradiation and saline injection.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Effects of the Hyperglycemic-Glycogenolytic Factor (HGF), Epinephrine and Insulin in Normal and Depancreatized Dogs.* (19715)

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During the course of experiments designed to study the action of certain insulin-free pancreatic extracts on the ketonemia and ketonuria of depancreatized dogs, it was noticed that the extracts had a marked hyperglycemic effect(1), probably because they contained the hyperglycemic-glycogenolytic factor (HGF). The extent of the hyperglycemia and its duration in different animals were extremely variable, however, even when the extracts were derived from the same batch of pancreas, were treated in the same manner and were injected under apparently identical conditions. Other experiments showed that the injection of epinephrine into depancreatized dogs with ketosis was also followed by a variable hyperglycemic response. The work described in this paper was undertaken to investigate the reasons for this variability.

Materials and methods. Well-fed mongrel dogs were depancreatized aseptically under sodium pentobarbital anesthesia. Following the operation, 100 ml of 0.9% saline, 100 ml of 10% glucose, 10 units of regular insulin (Lilly) and 30,000 of slow-acting penicillin were injected subcutaneously. This treatment was repeated on the first and, occasionally, on the second postoperative day. Penicillin injections were continued for 7 to 10 days. As soon as the animals started to eat, protamine zinc insulin (Lilly) was given instead of regular insulin. On the 7th postoperative day, the abdominal incision showed satisfactory healing and the stitches were removed. The dogs were kept in metabolism cages and fed the following daily ration: raw pancreas 100 g, sucrose 40 g, raw horse meat 500 to 700 g, depending upon the size of the animal. The urine was collected daily and the 24-hour glucose excretion was determined quantitatively; urinary ketone bodies were estimated according to an arbitrary scale (1+ to 4+) using Acetest§ tablets. The dogs were weighed once a week until the dietary and insulin requirements had been determined and

* Supported by a grant from Eli Lilly and Co.

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§ Acetest Tablets were donated by the Ames Co., Elkhart, Ind.

TABLE I. Maximum Blood Sugar Rise Following Intravenous Injection of "Crude" HGF Extract in Depancreatized Dogs with Ketosis.

Group 1—"Mild" ketosis				Group 2—"Severe" ketosis			
		Max blood sugar rise				Max blood sugar rise	
No.	Initial blood ketones, mg %	mg	% of initial value	No.	Initial blood ketones, mg %	mg	% of initial value
1	0	71.8	46.7	20	30.2	62.3	18.7
2	3.9	32.6	46.7	21	38.1	7.8	20
3	4.5	151.3	73.7	22	38.3	27.1	26.4
4	5.2	33.3	39	23	41.5	83.1	22.2
5	5.9	200.9	55	24	42.6	45.4	25.2
6	6.5	40	44.6	25	44.8	114.3	40
7	7	89.1	55.8	26	61.6	65.9	17.1
8	9.5	76.6	22.5	27	63.8	117.3	61.3
9	13.2	49.5	89.6	28	69.4	86.3	44.4
10	13.4	134.7	38.5	29	75.1	33	11.9
11	14.4	34.2	40.5	30	82.3	22.3	12.5
12	15.6	64.5	74.3	31	84	26	19.6
13	18.9	36.8	62.3	32	94.5	26.1	7.5
14	19	62.1	31	33	96.3	32	6.5
15	19.7	73.9	56.8	34	106.4	180.3	52.3
16	22.4	44	38.6	35	187.5	33.4	35.6
17	24.6	151.3	30.6				
18	26	48.8	18.9				
19	29.1	191.5	47.2				
Avg	13.6	81.5	48	Avg	72.2	60.9	26.3
		↑				↑	
		↑	P < .01				↑
			P < .01				

the animals had regained their preoperative weight, then at longer intervals. Protamine zinc insulin was administered at feeding time in doses sufficient to keep the urine acetone-free, the 24-hour glucose excretion below 10 g and the weight of the animal approximately constant. When an animal was needed for experiments, insulin was withdrawn until a strong (4+) ketonuria developed, usually 3 to 5 days. Quiet, well-fed mongrel dogs were used as controls. On the morning of an experiment the animals were not fed, control blood samples were taken, the extract containing HGF was injected intravenously and samples of blood were taken at various intervals of time for 4 hours. Blood ketone bodies were determined in duplicate following a modification of the method of Greenberg and Lester(2,3); blood glucose was determined in duplicate following the method of Folin and Malmrose(4). Urinary glucose was determined by means of Benedict's quantitative reagent. Two types of extracts containing HGF were used. The first one, which will be referred to as the "crude" HGF extract, was prepared as follows: 5.0 g of "12.5% salt cake," obtained by Eli Lilly and Company during the processs of insulin manufacture, were dissolved in 100 ml of 0.08 N NaOH; the solution was incubated at 39°C for 2 hours and the pH adjusted to 7.4 with dilute HCl. After filtration or centrifugation, the clear "crude" extract was injected intravenously in doses equivalent to 10 g of fresh pancreas per kilo of body weight. The second type of preparation was obtained by extracting lyophilized whole pancreas powder with liquid ammonia and purifying it partially by precipitation with 80% ethyl alcohol, according to a method described elsewhere(5). This preparation, dissolved in 10 ml of 0.9% saline, was injected in doses of 1 mg/kg and will be referred to as the "semi-purified" HGF. When indicated, the statistical significance of the results was calculated according to the method of Fisher(6) and expressed as "P", the probability that the results obtained might have been due to chance.

Results. The animals were listed in order of increasing concentration of ketones in the

TABLE II. Maximum Blood Sugar Rise Following Intravenous Injection of Epinephrine (.02 mg/kg) in Depancreatized Dogs with Ketosis.

Group 1—"Mild" ketosis				Group 2—"Severe" ketosis			
		Max blood sugar rise				Max blood sugar rise	
No.	Initial blood ketones, mg %	mg	% of initial value	No.	Initial blood ketones, mg %	mg	% of initial value
1	5.4	65.1	25	6	31.4	22.2	14.3
2	10.7	95.3	58.3	7	71.7	8	2.7
3	13.4	76.2	27.6	8	88.5	11.6	3.4
4	17.1	65.9	53.8				
5	18.2	46.5	25.6				
Avg	12.9	68.9	38.1	Avg	63.9	13.9	6.7

$P < .01$

$P < .01$

fasting blood and divided arbitrarily into 2 groups. Group 1 consisted of the animals with a ketonemia of less than 30 mg %, or with "mild" ketosis, Group 2 of the animals with a ketonemia of more than 30 mg %, or with "severe" ketosis. Table I shows that the average concentration of ketone bodies in the blood was 13.6 mg % in Group 1 and 72.2 mg % in Group 2. The response to the injection of the "crude" HGF extract was expressed as the maximal blood sugar rise reached during the 4-hour period of observation. It will be noted that the average hyperglycemic response was much greater in the animals with mild ketosis than in the animals with severe ketosis. The difference between these 2 average values is statistically highly significant ($P < 0.01$). Table II shows that similar results were obtained following the intravenous injection of epinephrine in doses of 0.02 mg/kg dissolved in 10 ml of 0.9% saline. Again the hyperglycemic response was inversely proportional to the initial concentration of blood ketones.

In another group of experiments, "semi-purified" HGF was injected intravenously into 10 normal dogs and 10 well controlled depancreatized dogs. These animals had received their last injection of insulin 24 hours before the experiment and had no ketonuria. Fig. 1 shows that the rise in blood sugar concentration produced by HGF was much greater in the controlled depancreatized dogs than in the normal ones. To determine whether or not, under the conditions of our experiments, depancreatized and normal dogs responded differently to the injection of insulin as well, regular insulin (0.1 unit/kg) was injected subcutaneously into 2 normal and 2 well controlled depancreatized dogs. Fig. 2 shows that insulin produced a more prolonged and pronounced hypoglycemia in the depancreatized than in the normal dogs. Thus the diabetic dog is more sensitive than the normal dog to the action of both HGF and insulin.

Discussion. The chemical and physiological properties of HGF have been reviewed(7,8). Like epinephrine, HGF stimulates the liver phosphorylase system and thus causes glycogenolysis and hyperglycemia(8,9). This effect depends upon an adequate supply of liver glycogen, and does not manifest itself fully when the glycogen stores have been depleted by fasting(9,10), uncontrolled experimental diabetes(10) and, possibly, certain forms of labile diabetes in humans(11). When insulin is withdrawn from a depancreatized animal, as the store of liver glycogen becomes progressively depleted, ketosis appears. This decreasing supply of liver glycogen is probably the cause of the inverse relationship between the concentration of ketone bodies in the blood and the rise in blood sugar produced by HGF and epinephrine. Glycogen depletion, however, cannot explain why the hyperglycemic response to the injection of HGF is smaller in the normal than in the depancreatized dog well controlled with insulin, as there is no reason to believe that these 2 types of animals have significantly different amounts of glycogen in

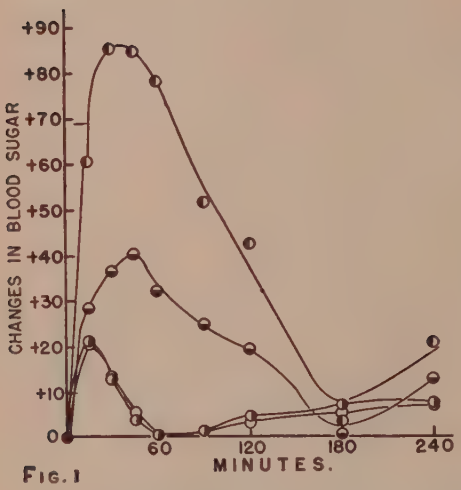

FIG. 1

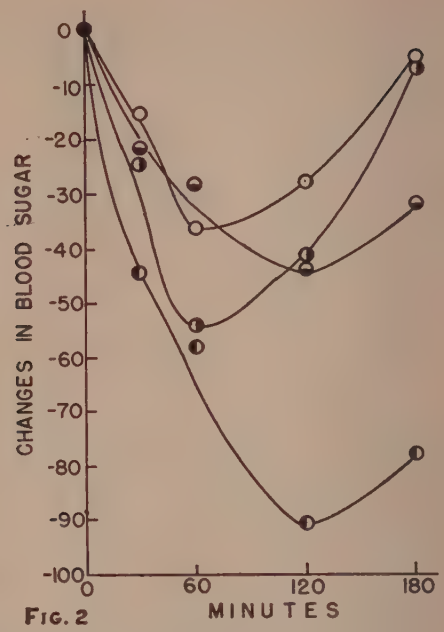

FIG. 2

FIG. 1. Hyperglycemia produced by intravenous injection of "semi-purified" HGF (1 mg/kg). Average of 10 normal and 10 depancreatized dogs without ketosis.

FIG. 2. Hypoglycemia produced by subcutaneous injection of regular insulin (.1 unit/kg). Average of 2 normal and 2 depancreatized dogs without ketosis.

their livers. A possible explanation is that, just as hyperglycemia produced by glucose administration stimulates the secretion of insulin by the pancreas of the normal animal (12-14), so the hyperglycemia produced by the injection of HGF might stimulate insulin secretion and thus, where a functioning pancreas is present, limit its own effect. Only in the depancreatized animal could HGF exert its full action. The observation that insulin has a greater effect in the diabetic than in the intact animal is in agreement with the results of others(15-17) and suggests that in the normal animal the reverse relationship between insulin and HGF may also exist: insulin hypoglycemia might cause the secretion of HGF and in turn be limited by it. In the depancreatized animal, on the other hand, HGF could not be readily secreted and insulin would be free to show its full effect. Thus, as several investigators have suggested (18-21), HGF would be a true anti-insulin hormone secreted by the pancreas itself and would have a function similar to that of other anti-insulin mechanisms(22). The secretion of the 2 pancreatic hormones would be mutually regulated by means of their opposing actions on the blood sugar. This hypothesis is being investigated further using cross-circulation experiments.

Conclusions. 1. The hyperglycemia produced by HGF and by epinephrine in the depancreatized dog with ketosis is greater in animals with mild ketosis than in animals with severe ketosis. 2. Since ketosis is believed to increase as liver glycogen decreases these results suggest that the hyperglycemic response to HGF and epinephrine depends upon the amount of liver glycogen present. 3. In the well controlled depancreatized dog without ketosis, on the other hand, the hyperglycemic effect of HGF is greater than in the normal animal, suggesting that in the presence of the pancreas, the action of HGF is limited by the secretion of insulin. 4. Similarly, in the well controlled depancreatized dog without ketosis, the hypoglycemic effect of insulin is greater than in the normal animal, suggesting that in

the presence of the pancreas the action of insulin is limited by the secretion of HGF. 5. It is suggested that HGF and insulin might be 2 mutually regulated pancreatic hormones and that their balanced secretion might be an important factor in the maintenance of a normal blood sugar concentration.

The guidance of Dr. A. H. Ryan in the statistical calculations and interpretation is gratefully acknowledged.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Measurements of Radioisotopes in Blood Applied to Determinations of the True Hematocrit.* (19716)

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A convenient and accurate technic for sample preparation for use in measurement of concentrations of soft beta-ray emitting radioisotopes in blood is required in clinical and experimental investigations. Solid samples, especially at or near "infinite thickness," prepared by the isolation of a precipitate of the radioactive ion give reproducible results(1,2). However, their preparation requires considerable time and the counting rate of the sample is lowered by absorption of beta-rays by the sample. Measurement of radioisotopes in liquid samples(3,4) exposed to a thin-window counter has been recommended. Such liquid samples, like thick solid samples, reduce the counting rate by self-absorption, thus necessitating the administration of large amounts of labeled isotopes. Liquid samples suffer the additional disadvantage of possible evaporation, unless covered during counting, and are easily spilled.

Rapid and simple methods for sample preparation, particularly applicable to blood containing beta-ray emitters of low energy, have been developed and tested. These procedures

* Supported by grants from the Research Grants Division, U. S. Public Health Service, and the Graduate School of the University of Minnesota. The radiocalcium and radiosodium used in the experiments were obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

have been found to give reproducible results and have few of the disadvantages of other methods. The procedures have a definite application, when I^{131} labeled albumin is employed, to the measurements of blood and plasma volumes and in the determination of the true hematocrit. The methods have also been successfully applied to the measurement of Ca^{45} and Na^{22} (a positron emitter) in plasma and blood. It is probable that they could also be employed for the measurement of other non-volatile electron emitters with maximum energies down to 0.15 mev.

The accuracy of relative corpuscle-plasma volumes obtained from the hematocrit has been repeatedly questioned on the score of the amount of plasma trapped in the corpuscle columns. The consensus(5,6) of the most consistent observations published prior to 1952 gave estimates of trapped plasma as 3-5% of the packed cell column. Maizels(7), however, found that only 2.2% of the packed cell column is plasma, while McLain and Ruhe(8) reported 4-17.9% of the cell column of defibrinated ox blood to be plasma. Leeson and Reeve(5) employed determinations of the dilution of I^{131} labeled serum proteins in blood by a complicated procedure and also made measurements of radioactivity in the packed cell columns and in the supernatant plasma. These workers reached the conclusion that about 5% of the cell column of heparinized human blood in the hematocrit tube is plasma. Jackson and Nutt(6) measured the concentration of T-1824 dye, added to whole blood, in the cell column and in the plasma and found that not over 2.2% of the cell column is plasma when the centrifugation is carried out at 4000 times gravity.

Methods. A 5-inch diameter circle was drawn on heavy paper attached to a plane surface of a transite board and the board was fixed in a level position as determined with a mechanic's bubble level. Whole blood was hemolyzed by the addition of an equal volume of water. The hemolyzed blood solution, or undiluted plasma, was treated with 0.08 ml of glycerol per milliliter and with 3-6 flakes of a solid surface tension depressant† and then shaken for several minutes to assure homogeneity of the solution. Glycerol was employed as a plasticizer to prevent brittleness, cracking, and flaking of the sample after drying. Half milliliter samples of the solutions were pipetted onto the center of 1-inch diameter copper planchets arranged on the perimeter of the circle mentioned above. A thin straight wire, such as hypodermic needle obturator, was used to distribute the sample evenly over the surface of the planchet. An infra-red radiant heating bulb was suspended approximately 4 inches above the center of the circle, and the samples were dried for one hour. The dried samples were firm and tough and adhered smoothly to the planchets. The radioactivity measurements were made with thin mica window Geiger-Mueller counters and care was taken to maintain a fixed geometric relationship between the sample and the counter window. All radioactivity counts were made within a statistical deviation of less than 1%.

Radiocalcium concentrations in whole blood and plasma were also determined by counting "infinitely thick" samples of calcium oxalate collected in previously described counting dishes(1). In these experiments protein-free filtrates of blood and plasma were prepared from trichloracetic acid and inert carrier calcium ion was added to the filtrates to give the required amount of calcium oxalate precipitate.

Hematocrit determinations were made with Wintrobe tubes centrifuged at 2000 r.p.m. (18.5 cm radius) until a constant packed cell column was obtained (1-1.5 hours). Duplicate measurements were made.

Results and discussion. The reproducibility of the results obtained by the methods of sample preparation described above was examined by counting several samples prepared from a single volume of plasma or blood containing I^{131} labeled albumin, Ca^{45}, or Na^{22}. The mean results, as counts per minute, with standard deviations of the means follow: 10 samples I^{131} albumin in plasma, 1034 ± 20.4; 10 samples I^{131} albumin in whole blood, $25{,}688 \pm 230$; 6 samples Ca^{45} in plasma, 3083

† Nacconol, a laboratory cleansing aid, manufactured by National Aniline Division, Allied Chemical and Dye Corp., Merchandise Mart, Chicago, was used.

± 31.6; 10 samples Ca^{45} in whole blood, 1983 ± 66.5; 6 samples of Na^{22} in plasma, 4936 ± 81.5; 10 samples of Na^{22} in whole blood, 1646 ± 20.1.

It was desired to compare the radioactivity measurements of whole blood and its plasma, each containing I^{131} labeled albumin, in order that the relative volumes of plasma and corpuscles in whole blood might be calculated. The greater solids content of whole blood results in the weight and thickness of the dried residues on the planchets from whole blood (diluted 1:1) being greater than those of the plasma residues. It was, therefore, necessary to examine the magnitude of radiation losses due to self-absorption in the whole blood residues. Five dilutions of whole blood containing I^{131} labeled albumin were made with water in such manner that half milliliter volumes contained the equivalent of 0.05-0.23‡ ml of whole blood when further diluted with 0.08 ml of glycerol per ml. The weights of the residues of these solutions, prepared as described above on tared planchets, varied from 56 to 94 mg of which 43 mg was due to glycerol. A plot of the counts per minute of the planchets against volume of blood represented in the samples, or against weights of the residues, gave a straight line. It is thus evident that self-absorption by the samples of I^{131} radiation is not a factor under the conditions of sample preparation described in this paper and that measurements of I^{131} activity in plasma and whole blood, by these technics, can be related directly.

The beta radiation of Ca^{45} exhibits considerable self-absorption in samples(9). The use of these technics with Ca^{45} would, therefore, require standard planchets prepared from plasma or whole blood, depending upon which fluid is to be studied. It would also be preferable to prepare the Ca^{45} standards concurrently with the experimental samples in order to control possible variations in degree of drying among the samples.

The plasma fraction of blood was calculated as the ratio of the radioactivity measurement of whole blood to that of an equal volume of plasma from the same blood sample. The apparently valid assumption is made that I^{131} from iodinated albumin added to blood remains in the plasma(5). The measurements were carried out with heparinized human blood treated with I^{131} labeled human albumin and also with the same blood after increasing its plasma volume. Plasma was collected from the original blood after addition of I^{131} albumin and added to aliquots of the original blood to give 2 blood samples with increased plasma fractions. The results compared with those obtained by the hematocrit are given in Table I.

The direct and derived results in Table I show that the hematocrit determination is liable to a considerable error due to plasma trapping in the cell column. This error decreases with increasing plasma volume (decreasing cell volume as, for example, in cytopenic or microcytic anemias). This observation may account for some of the variations as to plasma trapping in the hematocrit tube reported in the literature. On the basis of this observation it is suggested that ordinary hematocrit determinations applied to polycythemic blood considerably overestimate the true cell volume. Undoubtedly, the increasing centrifugal force(6) toward the closed end of the hematocrit tube is an important factor in reducing plasma trapping in short cell columns.

Although the results obtained with Sample C (Table I) by the 2 methods are virtually identical, it seems unlikely that the cell columns in the hematocrit tubes were devoid of plasma. It is possible that experimental errors, particularly in the hematocrit determination which became of increasing importance as the cell volume decreases, account for the agreement of these results.

The plasma volumes of blood samples and the intercellular plasma of the cell column of the hematocrit were also studied by measurements of Ca^{45} concentrations in whole blood and plasma. In this work, carried out before the sample preparation technics described in this paper were developed, Ca^{45} was measured in infinitely thick preparations of calcium oxalate as mentioned in the section on

‡ 0.23 ml is the volume of blood represented in the residue on the planchets prepared from whole blood by the routine technique.

TABLE I. Plasma Volumes of Blood Calculated from Hematocrit Determinations and from Concentrations of I^{131} Labeled Albumin in Blood and Plasma.

Material	Plasma vol. as % of blood vol. from Hematocrit	Plasma vol. as % of blood vol. from I^{131} albumin	Plasma vol. deficit by hematocrit (% of actual vol.) †	Trapped plasma as % of vol. of packed cell column
Original blood *(A)*	54.08	58.65 (6P,3B)*	7.8	9.9
A diluted with plasma *(B)*	69.31	70.72 (6P,4B)*	2	4.6
A further diluted with plasma *(C)*	74	73.97 (6P,8B)*	0	0

* These numbers denote the number of separate samples of plasma (P) and whole blood (B) counted.

† The actual volume taken to be that obtained from the radioactivity measurements (third column).

Methods. The plasma content of whole blood was taken to be the ratio of the Ca^{45} count of 1 ml of whole blood to that of an equal volume of plasma of the same blood.§

Small volumes of high specific activity Ca^{45} solutions were mixed with 18 heparinized whole blood samples (17 dog, 1 human). Plasma samples were promptly removed. The plasma content of each sample of whole blood was measured by the hematocrit and calculated from the Ca^{45} concentrations of duplicate samples of whole blood and plasma. The mean results of the 18 samples, with standard deviations, as percentage of plasma in blood follow: 55.66 ± 4.80 (by hematocrit) and 61.60 ± 4.12 (by Ca^{45}). In no case was the plasma fraction read from the hematocrit greater than that obtained by calculation from the Ca^{45} concentrations. The mean plasma volume deficit by hematocrit was $2.94 \pm 1.59\%$ of the whole blood volume. These results allow the calculation that, on an average, 7.1% of the packed cell columns in the hematocrit tubes was plasma. One sample of dog blood was treated with I^{131} labeled albumin and with Ca^{45}. The plasma fraction was estimated by each of the methods with the following results: 53.25% (hematocrit), 56.45% (I^{131} albumin), and 57.38% (Ca^{45}).

§ The assumption is made, as in the case with I^{131} labeled albumin, that Ca^{45} added to blood remains in the plasma. This assumption is justified by the very low, probably zero, calcium content of red cells (10). We shall show, however, in a subsequent report that detectable quantities of Ca^{45} are removed from plasma by red cells after 24-48 hours.

Summary. Simplified methods for sample preparation for use in estimation of I^{131}, Ca^{45}, and Na^{22} in blood and plasma are described. These methods will permit the administration of minimum amounts of I^{131} labeled plasma proteins in studies of blood and plasma volumes. The degree of plasma trapping in the centrifuged hematocrit was determined from measurements of I^{131} labeled albumin and Ca^{45} concentrations in equal volumes of whole blood and plasma. With normal human and dog blood 7-9% of the packed cell column in the hematocrit tube is plasma. The amount of plasma trapped decreases with decreasing cell column height. The practical implications of the last observation are mentioned.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Skeletal Calcium Turnover in Non-Growing Rats.* (19717)

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Singer and Armstrong(1) reported the existence in mature male rats of 2 kinds of skeletal calcium with markedly different rates of turnover. These forms of bone calcium were denoted as "mobile" and "fixed" fractions. Copp *et al.*(2) have also found evidence for the presence of a labile calcium fraction, amounting to approximately 15% of the total skeletal calcium, in the bones of young animals. Neuman and Mulryan(3) indicated that the mobile and fixed bone calcium fractions are the result of 2 distinct processes: (a) rapid ionic exchange of calcium for that on the surface of apatite crystals (the labile fraction), and (b) a slow recrystallization of the apatite crystals. The result of the latter process is to cause Ca^{45} to become situated in relatively inaccessible locations in the interior of the crystals, thus producing the stable bone calcium.

This study, with mature female rats, was carried out in order to eliminate the influence of skeletal growth on the results. Skeletal accretion has the effect of reducing calcium specific activity of bones(1) and might also render Ca^{45} in bones unavailable for removal by exchange through incarceration under layers of newly deposited bone or through deposition in older and more heavily mineralized Haversian systems(4,5). Unlike male rats, some female rats which can be selected by observation, attain a constant body weight. It was, therefore, assumed that such animals establish a nearly constant skeletal mass.

Experimental. Thirty female rats of the Sprague-Dawley strain whose weights had reached a plateau within the range 240-270 g were each given 3 intraperitoneal injections, at 2-hour intervals, of a high specific activity Ca^{45} solution. A total of approximately one million counts of Ca^{45} was administered. The animals were housed for 2 days following the injections in metabolism cages in order to permit the collection of the excreta and thus the estimation of the fraction of the injected Ca^{45} which was promptly excreted. The fraction of the injected dose remaining in the animals (Table I) after 2 days was assumed to have been fixed in large part in the calcified tissues. The animals were sacrificed in groups of 2-4 animals at one to 210 days after the administration of the radioisotope. Two days before the animals were to be sacrificed they were again placed in metabolism cages and urine collections were made over the 24 hours prior to sacrifice. During this 2-day period the animals were fed a diet devoid of calcium, but otherwise adequate, in order to eliminate contamination of the urine with spilled food calcium.

The femora, humeri, teeth, pelt,† and 3 lower lumbar vertebrae were obtained for analysis. The 4 epiphyseal ends of the femora of a single animal were pooled as one sample and the 2 diaphyses of the same bones were combined as another sample. The carcass residue, which consisted of the remainder of the animal, was ground in a meat grinder and then converted into an homogenate with a small amount of water in a Waring blendor. Three large aliquots of this suspension were taken for analysis and the results were averaged. All samples were dried, ashed at 700°C, dissolved in hydrochloric acid solution, and diluted to known volumes. The ash of the feces and of the homogenate samples required fusion with sodium carbonate at 900°C be-

* This study was supported by grants from the Research Grants Division of the U. S. Public Health Service and the Graduate School of the University of Minnesota. The radiocalcium employed in the experiments was obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

† At 28 days the pelt was found to contain negligible quantities of Ca^{45}. Accordingly this tissue from animals sacrificed at subsequent days was discarded without analysis. It appears probable that most of the Ca^{45} associated with the pelt is derived from saliva or urine.

TABLE I. Turnover of Calcium Labeled with Radiocalcium in Non-Growing Female Rats.

Day of sacrifice	No. of animals	Total calcium (mg)	% injected dose remaining: In animal after 2 days	In animal at sacrifice	In skeleton at sacrifice	Specific activities (% injected dose/mg Ca): Skeleton	Plasma	Urine
1	2	3000 (242)		62.9 (1.4)	53.8 (1.5)	.0199 (.0012)	.7631 (.0131)	2.2380 (.0810)
5	2	3203 (120)	62.7 (3.9)	51.7 (4)	44.6 (5.3)	.0148 (.0023)	.0960 (.0327)	.1585 (.0946)
10	2	2889 (166)	67.1 (2.3)	43 (3.7)	29.2 (.1)	.0109 (.0003)	.0778 (.0019)	.0760 (.0001)
20	2	2821 (157)	64.4 (2.8)	42.4 (2.7)	33.2 (2)	.0124 (.0004)	.0295 (.0012)	.0328 (.0070)
28	4	2936 (168)	64.1 (1)	38.2 (5.1)	29.7 (5.2)	.0122 (.0016)	.0250 (.0039)	.0291 (.0087)
40	2	3255 (143)	72.6 (1.4)	38.8 (.1)	32.9 (0)	.0106 (.0005)		.0154 (.0007)
50	2	3210 (288)	72.2 (6.2)	33.8 (.8)	30.8 (.9)	.0101 (.0012)		.0536 (.0055)
70	4	3254 (128)	74.2 (2.2)	35.7 (1.3)	34.8 (2)	.0112 (.0006)	.0106 (.0009)	.0116 (.0025)
90	3	2987 (40)	74 (4.3)	31.5 (7.7)	29.2 (6.5)	.0102 (.0022)	.0083 (.0017)	.0095 (.0015)
120	3	2943 (109)	73.2 (1)	23.3 (7.8)	22.8 (8.7)	.0081 (.0025)	.0090 (.0021)	.0069 (.0014)
154	2	3422 (107)	75 (1.9)	27.7 (2.4)	27.3 (2.3)	.0083 (.0004)	.0069 (.0014)	.0111 (.0021)
210	2	3223 (73)	72.8 (1.3)	23.6 (1.3)	23.3 (1.3)	.0075 (.0002)	.0062 (.0001)	.0063 (.0006)

Numbers within parentheses give the range of the results from the mean (2 animals) or average deviations from mean results (3 or 4 animals).

TABLE II. Calcium Specific Activities of Bones.

Day of sacrifice*	No. of animals	Specific activities (% inj. dose/mg Ca): Vertebrae	Femur epiphysis	Femur diaphysis	Humeri
1	2	.0204 (.0014)	.0188 (.0004)	.0074 (.0003)	.0136 (.0002)
5	2	.0180 (.0026)	.0173 (.0008)	.0088 (.0021)	.0150 (.0027)
20	2	.0154 (.0010)	.0154 (.0017)	.0078 (.0010)	.0146 (.0010)
210	2	.0105 (.0015)	.0080 (.0002)	.0060 (.0004)	.0065 (.0002)

Numbers within parentheses give range of results from the mean.
* To economize space, results for the selected days only are given.

fore solution in acid. The analytical methods and those used for the estimation of Ca^{45} have been described(1).

Results and discussion. The means of the results obtained from the animals sacrificed at each time interval are given in the tables. The total calcium content of the animals and the fraction of injected Ca^{45} present at sacrifice are the sums of total calcium and of Ca^{45} found in the individual components of the animal bodies. The results in the column headed "In skeleton at sacrifice" (Table I) exclude Ca^{45} in the teeth and pelt. The fractions of injected Ca^{45} given in this column are attributed to the skeleton because most of the body calcium of the edentulous and skinned carcass is in the skeleton. The specific activities of the skeletons were calculated by excluding the total calcium and Ca^{45} contents of the teeth and pelt.

The total calcium content of the animals exhibited some variation despite careful selection of animals of comparable age and weight. However, in contrast to the results obtained with male rats(1), there appears to have been no consistent increase of body calcium with increasing age of the animals, thus attesting

to the probable low order of bone accretion in individual animals during the experimental period. The percentage of the radioisotope retained after 2 days (difference between that injected and that excreted) was not closely correlated with the total calcium in the animals and showed an extreme variation between 62.7 ($\pm$3.9) and 75.0 ($\pm$1.9)%.

The percentage of injected Ca^{45} present in the animals and in the skeletons at sacrifice decreased rapidly during the first 10 days after the injections. From Day 10 through Day 90 the quantity of radioisotope retained in the skeletons remained nearly constant. It appears that a further decline in the fraction of Ca^{45} retained may have occurred after Day 90. In the light of our previous study(1) these findings are interpreted to indicate a rapid turnover and excretion of Ca^{45} from a labile bone fraction during which time a large part (*circa* one-third) of the Ca^{45} became incorporated into more stable locations from which it was only slowly released. These results are consistent with the concept(3) that calcium of the surface of bone crystals is rapidly exchangeable while that of the interior of crystals is made available for turnover more slowly by recrystallization.

The specific activities of the skeletons, plasma, and urines also furnish evidence of the existence of forms of skeletal calcium with different rates of turnover. The animals sacrificed on Day 1 excreted urine with a calcium specific activity many fold that of the plasma, due, undoubtedly, to early excretion of high specific activity calcium immediately after the injections and to a rapid decline during the first day in plasma calcium specific activity. By the 10th day the specific activities of the plasma and urine became equal, within the limit of experimental variation, and remained so throughout the rest of the experiment. At Day 10 the specific activity of the plasma was 7 times greater than that of the skeleton. Only after Day 50 did the plasma specific activity fall to values equal, within the experimental variation, to those of the total skeletal calcium. It is, therefore, suggested that through Day 50 a relatively rapidly exchangeable form of skeletal calcium was removed by excretion or incorporation into a stable form and attained equilibrium with the calcium of the plasma. Beginning with Day 70, Ca^{45} in the plasma was maintained by the slow turnover of the more stable form of skeletal calcium.

In the previous study(1) with male rats whose skeletal calcium nearly doubled during the experimental period, 42-45% of the injected Ca^{45} became incorporated in the stable bone fraction. In the present study with non-growing female animals, not more than about one-third of the administered Ca^{45} was present in the stable bone fraction through Day 90 and this quantity declined to about one-fourth of the injected radioisotope from Day 120 through Day 210. It is suggested that the higher fraction of administered Ca^{45} initially incorporated by the skeletons of male rats and the greater quantity of the radioisotope present in the "fixed" bone compartment of the male animals were due to their greater bone accretion. In the growing animal Ca^{45} is accumulated in bones not only by exchange but in addition by deposition of bone salt from a medium containing the radioisotope.

The results presented in Table II show that various skeletal components differ in their abilities to accumulate Ca^{45} and in turnover of the isotope. The vertebrae, femur epiphyses and humeri accumulated per unit weight of calcium relatively more Ca^{45} than the femur diaphyses. Also, the femur diaphyses retained through Day 210 about 80% of their original Ca^{45} content while the other bones allowed the removal of about 50% of the radioisotope present at Day 1. These findings indicate that the quantitative features of calcium turnover by exchange and recrystallization vary in different bones. They are also in agreement with the work of Comar, Latz, and Boyd(6) who also found that anatomical location in a given bone influences its turnover of calcium.

Summary. Total calcium and radioactive calcium contained in selected skeletal components and in the entire bodies was determined in non-growing female rats at intervals up to 210 days after administration of the radioisotope. Evidence for fractions of skeletal calcium with markedly different rates of

turnover was obtained from the changing rates with time in removal of radiocalcium from the skeleton and from comparisons of calcium specific activities of the skeleton and plasma of the animals sacrificed at increasing times after administration of the radiocalcium. In this study the effect of skeletal accretion was minimized. The results confirm those previously obtained when skeletal growth was a possible factor influencing the results. These findings and interpretations are consistent with the concept that the bone salt exchanges the calcium of crystal surfaces rapidly and that of the interior of crystals more slowly by recrystallization. The specific activities of different bones and the change in specific activity of the bones with time indicate that anatomical location of bone influences its calcium turnover.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

A Rapid Method for Distinguishing D-Glucosamine from Galactosamine in Biological Preparations. (19718)

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In the course of a study of the constituents of a polysaccharide isolated from *Shigella flexneri*(1), it became necessary to be able to distinguish between the 2 naturally-occurring aminosugars, glucosamine and galactosamine. These substances have very similar R_f values and do not separate well in a number of solvent systems(2). The N-2,4 dinitrophenyl derivatives separate only slightly better(3) and methods for isolating derivatives involve more laborious procedures with relatively large amounts of aminosugars(4,5).

Brown reported the phosphorylation of D-glucosamine by hexokinase, and adenosinetriphosphate (ATP), the product being identified as D-glucosamine-6-phosphate(6). As is shown below, galactosamine does not function as a substrate for hexokinase. It is therefore, possible to differentiate between these aminosugars; and, in cases where these are the only 2 aminosugars present, the relative amounts of each may be determined.

Materials. The aminosugars used in the experiment described by Fig. 1 were partially purified from crude hydrolysates of the polysaccharide of *Sh. flexneri*(1) and bovine tracheae(7,8) by passage over a cation exchange resin and elution with HCl. The yeast hexokinase was also only partially purified(9).

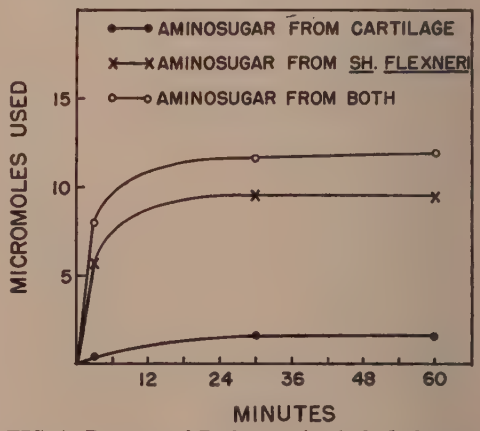

FIG. 1. Presence of D-glucosamine in hydrolysates of the polysaccharide of *Sh. flexneri*. The reaction mixture contained 33 micromoles ATP, 13 micromoles galactosamine from chondroitin sulfuric acid or 11 micromoles aminosugar from the polysaccharide of *Sh. flexneri*, or both, .006 M $MgCl_2$ and yeast hexokinase in .06 M veronal buffer, pH 8. Incubated at 30°C. The utilization of aminosugar corresponds to the D-glucosamine content of the reaction mixture.

Method. D-glucosamine is phosphorylated in the presence of ATP and hexokinase. Any unreactive aminosugar is determined after removal of glucosamine-6-phosphate by treatment of the reaction mixture with $ZnSO_4$ and $Ba(OH)_2$. This is necessary since the phosphorylated aminosugar also gives the color reaction of the free aminosugars.

The reaction mixture of the data in Fig. 1 contained the following:

ml	
4	.1 M veronal buffer, pH 8
.4	.1 M $MgCl_2$
.9	aminosugars (Fig. 1)
.3	.11 M ATP

The initial concentration of aminosugar was determined in the supernatant fluid obtained after adding a 1 ml aliquot of the above mixture to 1 ml of $ZnSO_4$ and then adding 1 ml $Ba(OH)_2$(10). 0.2 ml of hexokinase was added to 4 ml of the remaining mixture to start the reaction which was incubated at 30°C. One ml samples were fixed with $ZnSO_4$ and $Ba(OH)_2$ at timed intervals. The supernatant fluid which is free of protein and phosphorylated compounds was used for the determination of free aminosugars by the colorimetric method of Elson and Morgan(11).

Results. The galactosamine preparation showed a utilization amounting to only 10-15% of the total aminosugar added, whereas 85% of the aminosugar obtained from the specific polysaccharide of *Sh. flexneri* was phosphorylated. The limited disappearance of aminosugar from the galactosamine preparation indicates that some D-glucosamine may have been present in the chondroitin sulfuric acid from which the galactosamine was isolated. It may also be seen that the presence of galactosamine did not prevent the phosphorylation of glucosamine obtained from the hydrolysate of the polysaccharide.

Discussion. It is possible to carry out determinations on crude hydrolysates of biological materials provided an excess of ATP is added so that it does not become limiting by the phosphorylation of glucosamine or of other sugar residues such as glucose in the hydrolysates. In cases where acid hydrolysis is not desirable, ATP-destroying enzymes, if present in amounts sufficient to prevent the maintenance of an excess of ATP during incubation with hexokinase, may be eliminated by deproteinization and neutralization of the sample before adding it to the test system.

The disappearance of as little as 0.1 micromole of glucosamine per color test may be detected easily when it is the only aminosugar present. With galactosamine as the only aminosugar, no disappearance of color equivalents occurs during incubation with hexokinase and ATP. Mixtures of the 2 aminosugars may be accurately analyzed for both components provided at least 10% of the total aminosugar is D-glucosamine. Because of the non-utilization of galactosamine during treatment with hexokinase, it is easier to detect small amounts of this aminosugar in the presence of large amounts of D-glucosamine than it is to detect a small decrease in color due to the presence of a trace of D-glucosamine in the presence of a large amount of galactosamine.

Summary. An enzymatic method is described for distinguishing D-glucosamine from galactosamine. The method is based on the fact that glucosamine is phosphorylated by ATP in the presence of hexokinase, whereas galactosamine is not. Application of the procedure for the assay of biological preparations is illustrated by the demonstration of D-glucosamine in hydrolysates of a polysaccharide isolated from *Shigella flexneri.*

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Nucleic Acids and Their Components in Tumor-Bearing Mice during Growth and Regression of Tumors.* (19719)

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In recent years, a great deal of attention has been directed to the part played by nucleic acids in neoplastic growth. Several investigators(1,3,5,8,13,14) have presented evidence indicating a high nucleic acid content for tumor tissues. It has been shown recently in this laboratory(2,7,9) that there is an increase in the nucleic acid content of the liver, kidney, and lung of mice bearing a transplantable sarcoma.

Bischoff *et al.*(21) reported that a synthetic diet containing vitamins of the B complex other than B_6 produced a marked decrease in the growth rate of sarcoma 180 in Marsh-Buffalo mice, and addition of vit. B_6 increased it again. On the other hand, Morris(22) found that the growth of spontaneous mammary adenocarcinoma in strain C3H mice was decreased by extreme deficiency of pantothenic acid and riboflavin produced rapidly during a short period, and was not affected by extreme deficiency of pyridoxine.

Stoerk(15) reported the regression of lymphosarcoma implants in mice which were receiving desoxypyridoxine on a pyridoxine deficient diet. This experiment was repeated by us with a view of making a comparative study of the nucleic acids and their purine constituents in mouse tissues during tumor regression. Because of the pronounced changes in desoxyribose nucleic acid (DNA) which were found during tumor regression in the spleen, the pyrimidines were also determined in this tissue.

Experimental. Male mice of the C3H strain of approximately 5 weeks of age were employed in this experiment. A mouse lymphosarcoma,† 6-C3H-ED, was transplanted subcutaneously with a biopsy needle into the right pectoral region of the mice. The animals were placed on a stock diet (Purina Laboratory Chow) and were given ordinary tap water to drink.

The tumor implants were allowed to grow from 8 to 11 days at which time a number of animals were sacrificed, and their tissues separately pooled into groups of 4 animals each. The remaining animals were divided into 2 groups, experimentals and controls.

The experimental animals, designated "B_6 deficient," were placed on a diet deficient in vit. B_6. The composition of the diet was as follows: Vitamin-free casein (Labco), 25 g; sucrose, 53 g; Crisco, 10 g; lard, 5 g; salts,‡ 5 g; Ruffex, 2 g; thiamine, 1 mg; riboflavin, 1 mg; choline, 150 mg; calcium pantothenate, 10 mg; vit. A,§ 6,750 I.U.; alpha-tocopherol, 4 mg; and vit. D,‖ 500 I.U. These animals also received desoxypyridoxine¶ in their drinking water, at a level of 300 μg per ml.

The controls, designated "B_6 sufficient," were placed on the same diet except that vit. B_6 was added at a level of 20 mg per 100 g of diet. They also received 300 μg of desoxypyridoxine per ml in their drinking water.

A number of animals from each group were sacrificed at different stages of tumor growth as shown in the figures. The experiments were carried out in 3 different sections and with a total of 126 mice. Ten mice on the vit. B_6 deficient diet and 6 mice on the vit. B_6 sufficient diet died during the course of the experiment. These mice were discarded.

* This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research.

† Obtained from the Jackson Memorial Laboratory, Bar Harbor, Me.

‡ Osborne and Mendel.

§ Given in the form of a concentrate (1 000 000 I.U./g) supplied by Nopco Chemical Co., Harrison, N. J.

‖ Given in the form of Drisdol (Winthrop-Stearns Inc., New York).

¶ Kindly supplied by Merck and Co., Rahway, N. J. through the courtesy of Dr. Karl Folkers.

TABLE I. Tumor Development in Mice Receiving Desoxypyridoxine on a Vit. B_6 Deficient Diet and on a Vit. B_6 Sufficient Diet.

a. Treatment—desoxypyridoxine plus a vit. B_6 deficient diet

Before treatment				After treatment			
No. of animals	Body wt (g)	Tumor diam. (cm) Avg	Range	No. days on treatment	Body wt (g)	Tumor diam. (cm) Avg	Range
4	20	1.6	(1.3–1.7)	7	16	1.3	(1.2–1.3)
6	22	3	(2.6–3.2)	7	17	.6	(.4– .9)
5	23	2.2	(2 –2.5)	8	19	.7	(0 –1)
4	19	1.5	(1.4–1.7)	9	15	.3	(0 – .7)
3	23	2.1	(1.9–2.2)	10	19	.4	(0 –1)

b. Treatment—desoxypyridoxine plus a vit. B_6 sufficient diet

Before treatment				After treatment			
No. of animals	Body wt (g)	Tumor diam. (cm) Avg	Range	No. days on treatment	Body wt (g)	Tumor diam. (cm) Avg	Range
6	19	1.5	(1.3–1.7)	7	26	3.6	(3.4–4)
8	21	3	(2.9–3.1)	7	26	3.9	(3.6–4.6)
5	22	2.1	(1.9–2.3)	8	29	4.3	(3.8–4.7)
6	18	1.5	(1.2–1.6)	9	25	4.3	(3.8–4.5)
4	24	2.2	(2 –2.5)	10	27	4.1	(3.7–4.7)

All animals were sacrificed by decapitation and the tissues upon removal frozen immediately with dry ice. The tissues (liver, kidney, lung, spleen, and tumor) were separately pooled into groups of 4 or 5 and homogenized in a glass homogenizer using distilled water at 0°C. All homogenates were approximately 10-15% suspensions.

Aliquots of 2 ml were removed for nucleic acid analysis, pyrimidine analysis, and dry weight determinations. For the latter, all samples were dried at 105-110°C to constant weight. The nucleic acids were extracted by the trichloroacetic acid method of Schneider (12). One ml of the extract was utilized for the estimation of ribose nucleic acid (RNA) according to the method of von Euler and Hahn(4) and 0.5 ml for the estimation of desoxyribose nucleic acid (DNA) by Stumpf's (16) method. The nucleic acids in the remaining extract were hydrolyzed by heating with 1 N H_2SO_4 in a boiling water bath for 2 hours, under which conditions we have found that the purines (adenine and guanine) are completely liberated. Adenine was estimated in the hydrolysate by Woodhouse's(18) method and guanine by the method of Hitchings(6).

The pyrimidines (uracil, cytosine, and thymine) in the spleen were determined by paper chromatography according to the procedure of Vischer and Chargaff(17) with the modifications used by Cerecedo *et al.*(2). Wyatt's (19) solvent was used to develop the chromatograms, making possible the complete separation of the pyrimidines with one solvent.

Analyses were also performed on normal animals covering the entire experimental period.

Results. In Table I are presented data on the average tumor diameter before and after the treatment specified, the ranges of the tumor diameters, and body weights. It will be seen that the tumor grows rapidly in mice receiving desoxypyridoxine on a vit. B_6 sufficient diet. On the other hand, the tumor regresses in mice receiving desoxypyridoxine on a vit. B_6 deficient diet. The regression is as great as 100% in certain cases after 8 days treatment with desoxypyridoxine on a vit. B_6 deficient diet. These data clearly confirm Stoerk's(15) observation that lymphosarcoma implants regress in mice which receive a pyridoxine deficient diet and in addition desoxypyridoxine.

In one group of the vit. B_6 deficient animals the average loss in body weight was 5 g, whereas in the other 4 groups it was 4 g. On the other hand, the animals receiving desoxypyridoxine on a vit. B_6 sufficient diet gained weight. The vit. B_6 sufficient animals ate on the average 2.3 g of food per day while the vit. B_6 deficient animals ate 1.5 g of food per day. However, it must be remembered that

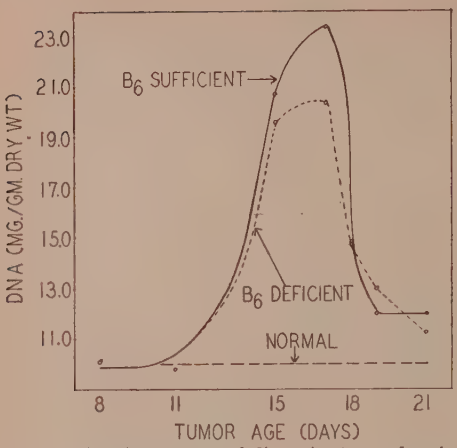

FIG. 1. DNA content of liver in tumor-bearing mice a) receiving vit. B_6, and b) deficient in vit. B_6.

caloric restriction on a tumor already established is relatively small. The major effect of caloric restriction is on the genesis or incidence of a tumor rather than on the growth of a tumor already firmly established(20).

The effects of desoxypyridoxine on a vit. B_6 deficient diet in the mouse seem to be specific. That is, it is not merely a general depression of body weight and a decrease in the rate of tumor growth. The data presented by Stoerk(15) and the data presented in Table I of this paper confirm this point.

In Fig. 1 the concentration of DNA in the liver (expressed in mg/g dry weight) against the tumor age in days has been plotted. The horizontal dotted line indicates the normal level of DNA in the liver. It may be seen that in mice on a vit. B_6 sufficient diet there is a 100% increase in DNA when the tumor is 15 days old. This increases further and is maximal when the tumor is 17 days old, but then decreases steadily and returns almost to normal at 21 days. The same trend is shown by animals on a vit. B_6 deficient diet except that the maximum at 17 days is not as high as that of the animals on a B_6 sufficient diet. RNA shows a similar trend (not shown in the figure), but again reaches a higher level in the vit. B_6 sufficient animals. In both the B_6 sufficient and deficient animals, the increases in RNA were not as great as those in DNA. This was evident from the RNA/DNA ratio which reached its lowest level when the tumor was 17 days old but then increased again returning almost to normal at 21 days. Adenine and guanine followed essentially the same trend as the nucleic acids. However, the increases in guanine were greater than those in adenine so that the ratio of guanine to adenine reached a maximum at 17 days of tumor growth.

Fig. 2 shows the concentration of RNA and DNA in the lung. The vit. B_6 sufficient animals again show an increase in DNA which is maximal at the 17th day of tumor growth. The deficient animals show a maximum in DNA at 15 days which then falls to normal at 21 days. RNA increases to a maximum at 17 days in the B_6 sufficient animals, and then drops almost to normal at 21 days. The B_6 deficient animals show the same trend but actually return to normal at 21 days. In each case, the nucleic acids reach a higher level in the vit. B_6 sufficient animals. Adenine and guanine show the same pattern as the nucleic acids (not shown on the figure).

In Fig. 3 the changes in RNA and DNA in the kidney are shown. The vit. B_6 sufficient animals show an increase in DNA which is maximal when the tumor is 17 days of age, and then drops. The deficient animals show

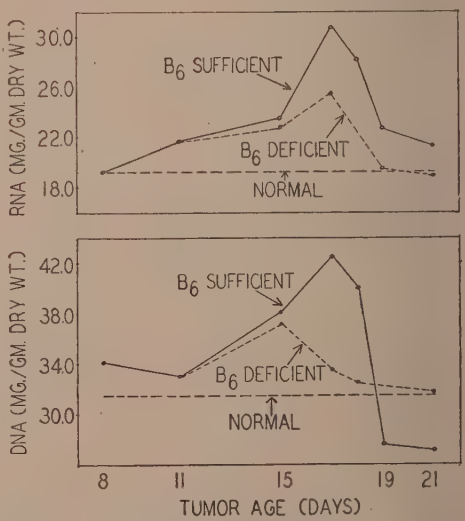

FIG. 2. DNA and RNA contents of lung in tumor-bearing mice a) receiving vit. B_6, and b) deficient in vit. B_6.

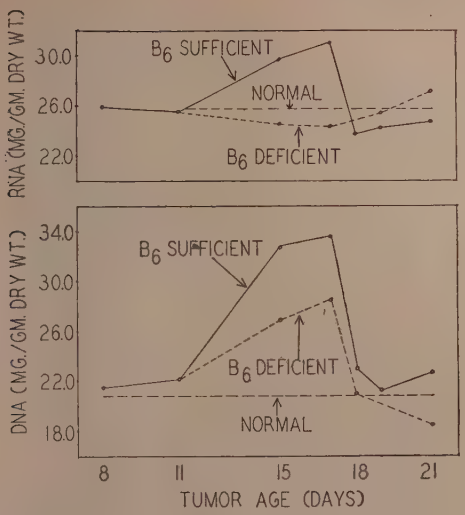

FIG. 3. DNA and RNA contents of kidney in tumor-bearing mice a) receiving vit. B_6, and b) deficient in vit. B_6.

a similar trend except that the increase in DNA is not as great. Adenine and guanine follow essentially the same pattern.

RNA does not rise above normal in the kidney of the deficient animals. On the other hand, it increases in the vit. B_6 sufficient animals to a maximum at 17 days and then falls. In addition to the liver and lung, therefore, the kidney presents further evidence that the nucleic acids reach a higher level in the vit. B_6 sufficient animals than in the deficient animals.

In the sarcoma (figure not shown), DNA and RNA reach a maximum at the 17th day of tumor growth. These are higher in the vit. B_6 sufficient mice. In each case, the rate of increase of DNA is greater than that of RNA up to the 17th day. Both DNA and RNA then decrease until at 21 days their values are comparable to the values at 11 days of tumor growth. Again, the rate of decrease of DNA is greater than that of RNA and at 21 days the ratio of RNA/DNA is comparable to that at 11 days.

In Fig. 4 the data for DNA and thymine in the spleen are given. Vit. B_6 sufficient animals show a maximum in DNA when the tumor is 17 days old. However, this value is close to normal. It then drops to a value approximately 20% below normal at 19 days.

The deficient mice show a quite different effect. In these animals, DNA drops quite sharply in the spleen. At 19 days this value is as low as 29.8 mg/g dry weight which is approximately 70% below normal. This is substantiated by a 70% drop in thymine from a value of 10.0 mg/g dry weight when the tumor is 11 days old to a value of 3.0 mg when the tumor is 19 days old. Cytosine, guanine, and adenine were found to exhibit a similar drop.

RNA remains close to normal in the spleen during tumor growth and regression, and this was verified by the fact that uracil remains fairly constant.

Discussion. The increase in nucleic acids in the various tissues during neoplastic growth is in agreement with previous results obtained in this laboratory. The data reported in the present paper indicate that there is an increase in the nucleic acids and their purine constituents in the liver, kidney, and lung during growth of a transplanted lymphosarcoma in C3H mice. These increases in the concentration of nucleic acids in the tissues of the host point to a stimulation of the nucleic acid

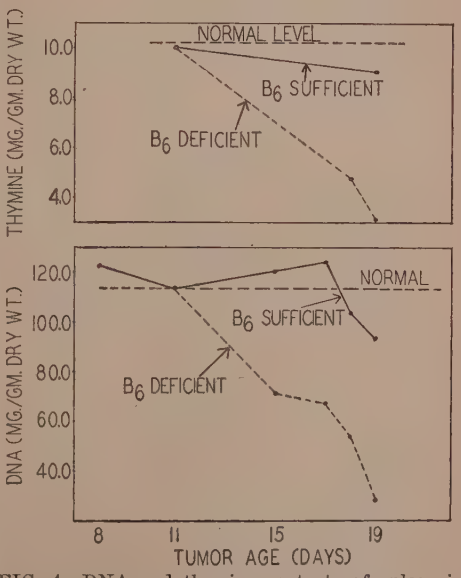

FIG. 4. DNA and thymine contents of spleen in tumor-bearing mice a) receiving vit. B_6, and b) deficient in vit. B_6.

metabolism during neoplastic growth.

It is of interest to point out that the DNA concentration at its highest level in the mouse lymphosarcoma (93.9 mg/g dry weight) is higher than that of Sarcoma 180 (49.1 mg/g dry weight)(7), and melanoma S-91 (61.7 mg/g dry weight)(2). This may be due to the high cellularity of the lymphosarcoma.

The fact that the nucleic acids reached a higher level in mice receiving desoxypyridoxine on a vit. B_6 sufficient diet than in mice receiving desoxypyridoxine on a vit. B_6 deficient diet suggests that desoxypyridoxine may act as an inhibitor in nucleic acid synthesis.

The pronounced drop in DNA in the spleen is very interesting. It has been suggested that pyridoxine is essential for the maintenance of the lymphocytes(10,11) of lymphatic tissue. Since the lymphocytes are rich in nucleic acids, it is possible to conceive that pyridoxine may play a fundamental part in nucleic acid synthesis possibly as a cofactor and thus maintain the normal lymphocyte level.

Summary. 1. The concentration of ribose nucleic acid (RNA), desoxyribose nucleic acid (DNA), adenine, and guanine of liver, lung, kidney, and spleen of normal C3H mice and those bearing a transplantable mouse lymphosarcoma at different stages of tumor development has been determined. The mice bearing the tumor were divided into 2 groups; one group received desoxypyridoxine on a vit. B_6 deficient diet, while the other received desoxypyridoxine on a diet rich in vit. B_6 to counteract the effect of the anti-vitamin. In addition to DNA, RNA, adenine, and guanine, the pyrimidines (uracil, cytosine, and thymine) were also determined in the spleen. 2. The data presented indicate that there is an increase in the nucleic acids and their purine constituents in the liver, kidney, and lung during the growth of the tumor. 3. The nucleic acids reached a higher level in mice receiving desoxypyridoxine on a vit. B_6 sufficient diet than in mice receiving desoxypyridoxine on a vit. B_6 deficient diet. 4. Mice receiving desoxypyridoxine on a diet high in vit. B_6 show a maximum value in DNA (which is close to normal) in the spleen on the 17th day of tumor growth which then drops to approximately 20% below normal at 19 days. In mice receiving desoxypyridoxine on a diet deficient in vit. B_6, DNA in the spleen drops quite sharply. At 19 days, this drop is over 70% below normal and this is followed by a similar drop in thymine, cytosine, guanine, and adenine. RNA does not vary to a significant extent in the spleen, and this is also true of uracil. 5. The changes in nucleic acids are followed generally by similar changes in adenine and guanine.

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Received June 12, 1952. P.S.E.B.M., 1952, v80.

A Simple Blood Flow Meter.*† (19720)

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While assembling equipment for the Starling heart-lung preparation, the problem of constructing an adequate blood flow meter became apparent. We arbitrarily established the following requirements: 1) accuracy of about 5%, 2) direct recording of flow against time, 3) linear response or a simple algebraic function thereof, 4) relative independence from viscosity changes, 5) immediate response to changes in flow, 6) simplicity of construction.

A recording meter was ultimately devised to meet these criteria using a test tube, a spring, a heart lever, a funnel and a suitable chassis to hold the components together as shown in Fig. 1. It was constructed as follows: A hole approximately 3 mm in diameter was blown in a pyrex test tube (22 x 175 mm). The test tube was suspended by a wire ring attached to an open coil stainless steel spring (wire diam. 0.009 in., O.D. 0.078 in., length 2.5 in.). One end of a fine lever was coupled to the lower end of the spring, the other traced a record on a kymograph. The lever arm ratio was approximately 10:1. The inflow was delivered through a tube running the length of the vessel. The outflow was collected in a funnel supported directly beneath the orifice. All glass parts were siliconed.

The theory upon which this meter is based depends on a consideration of the conservation of energy. By equating the potential energy of a unit volume of fluid at the top of a vessel to the potential energy of a unit volume at an orifice in the bottom of this vessel, it becomes obvious that the height of the fluid column is proportional to the square of the outflow velocity.‡ Since the effective orifice is essentially constant, the rate of outflow is directly proportional to the velocity; thus height is directly proportional to the square of the flow. For any given inflow rate, the fluid height will increase until the outflow rate becomes equivalent. At equilibrium, the fluid height remains constant. Range is determined by the vessel height and the diameter of the orifice. Thus, to measure flow, one need only measure fluid height in such a vessel. Since the vessel is of uniform diameter, height is a linear function of weight. Therefore by suspending the vessel on a spring, one may measure the deflection of an appropriately coupled lever and find it proportional to the square of the rate of flow. For electronic recorders, a strain gauge may be used instead of a spring.

Deviations from theory, due to the physical

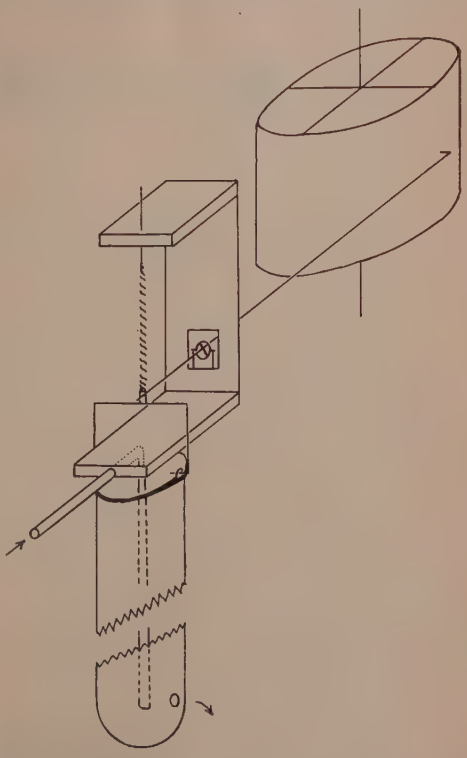

FIG. 1. Diagram of flow meter construction. The orifice is placed at one side of the bottom, in order to avoid the jet of incoming fluid. The funnel below the orifice is not shown.

* Presented in part at the Federation Meetings at New York April, 1952.

† This investigation was supported in part by a grant from the Squibb Institute for Medical Research.

‡ $mgh = \frac{1}{2} mv^2$; $kh = v^2$.

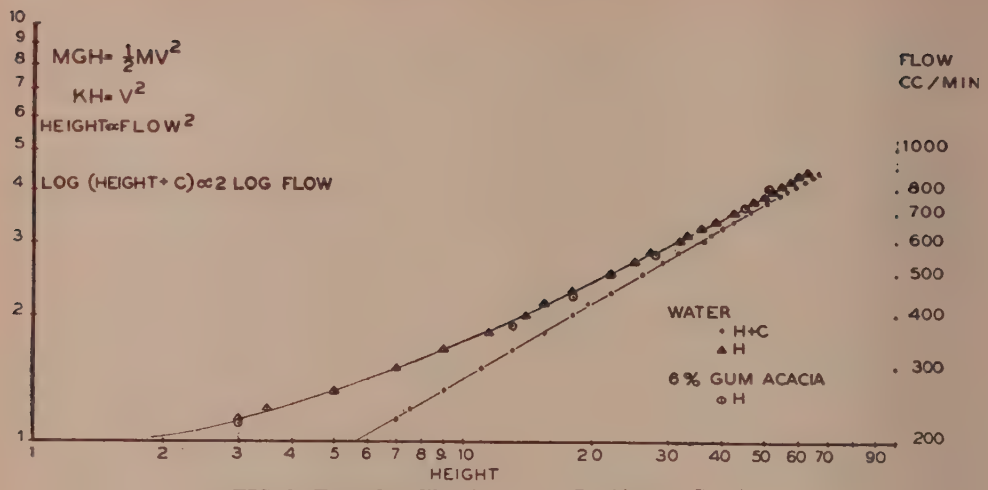

FIG. 2. Example calibration curve. In this case C = 4.

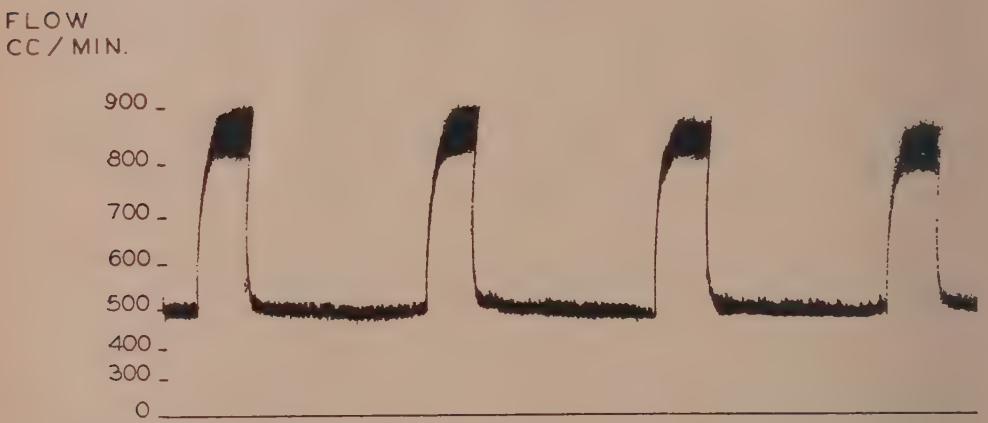

FIG. 3. Flow meter tracing showing response of cardiac output, in the Starling heart-lung preparation, to suddenly raising the venous reservoir 25 mm for 2 minute intervals every 10 min. The initial flow of 510 cc per min increased to 870 cc per min on the first test. Thirty min later, the response was from 505 cc per min to 830 cc per min indicating the amount of spontaneous failure.

properties of fluids, may be minimized by keeping the diameter of the vessel as large as possible and the orifice as short (that is to say the thickness of the vessel wall as thin) as possible. The meter is totally inaccurate when the fluid height falls to within several millimeters of the orifice.

Calibration was accomplished by allowing water to flow through the meter at various constant rates and measuring the volume collected in one- or 2-minute intervals. A linear calibration curve was constructed by plotting the vertical pointer height against the square of the flow. For convenience in reading the calibration curve the log height was plotted against 2 log flow, using several points to draw a line on log-log graph paper as illustrated in Fig. 2. This plot was linear only if it passed through the origin. Therefore, in order to obtain a linear calibration it was necessary to find the intercept, C, and plot 2 log flow against log (height + C). C was then added to the observed height before reading flow from the graph. In all cases, the

calibrated points were within about 5% of the best fitting line. When calibration lines using water were compared with calibration lines using 6% gum acacia (relative viscosity 7.5), the difference was never greater than 3%.

In a somewhat similar manner, Stephenson (1) has constructed a flow meter in which the height of a fluid column was related to the rate of discharge through a capillary. Due to pneumatic coupling to the recording device, this meter would seem to be sensitive to temperature and barometric pressure changes. The meter would also seem very sensitive to changes in viscosity and surface tension because of its dependence on capillary flow and the presence of drop formation at the orifice. Due to these factors, and to the presence of turbulent flow, there is no simple mathematical expression for its performance.

In our arrangement of the heart-lung apparatus, the meter was placed at the end of the arterial system, the outflow from the funnel going directly to the venous reservoir. A sample blood flow recording is shown in Fig. 3.

Since small changes in flow may be obscured due to frictional resistance of the writing stylus on the paper, it is suggested that a small electric vibrator be placed on the flow meter support. When the flow is pulsatile, as in the heart-lung preparation, the vibrator is unnecessary.

When the vessel diameter is increased, a larger volume increment is required in order to reach equilibrium and the response time is therefore lengthened. This results in a reduction in the oscillations due to transient changes and the meter approximates, to a finer degree, mean flow.

Summary. An inexpensive direct recording flow meter, relatively independent of viscosity changes and with an accuracy of about 5% was constructed. Its chief advantage lies in its extreme simplicity. The calibration curve, closely approximating a parabolic function, was reduced to a straight line by plotting log height against 2 log flow on log-log graph paper.

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Received June 23, 1952. P.S.E.B.M., 1952, v80.

Transport of Radioactive Sodium Across the Synovial Membrane of Normal Human Subjects.* (19721)

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(Introduced by William S. McCann.)

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Any investigation of physiological function of the synovial membrane of joints is limited by suitable technics for the study of this relatively inaccessible structure. The availability of a variety of isotopes obviates some of these difficulties. The present investigation was designed to study the sodium clearance of the synovial membrane by means of radioactive sodium.

A group of 35 healthy adults were injected in the knee synovial space with Na^{24} and the clearance rate determined. The results of this study of normal subjects reveal that significant differences are encountered between normal men and women. The present report describes these variations.

Method. Radioactive sodium (Na^{24}) was obtained from the Oak Ridge National Laboratories as $Na^{24}{}_2CO_3$. It was converted to $Na^{24}Cl$ and suitably diluted to produce an 0.85% sodium chloride solution with an adjusted pH of 7.3. The concentration of Na^{24} was 30-40 μc in each milliliter. After local novocaine anesthesia a 20-gauge needle was inserted under the lateral inferior border of the patella into the synovial space of the knee. Two milliliters of the sterile radioactive

* Aided by grant from the Masonic Foundation for Medical Research and Human Welfare.

TABLE I. Rate of Absorption of Na^{24} from Synovial Space of Knee.

	No.	% remaining after 30 min
Men	14	$\bar{X} = 48.2 \pm 2.3$
Women (total)	24	$\bar{X} = 40.8 \pm 1.8$
1st 10 days	7	$\bar{X} = 35.9 \pm 3.3$
2nd 10 "	10	$\bar{X} = 42.1 \pm 2.8$
3rd 10 " of menstrual cycle	7	$\bar{X} = 43.8 \pm 3.3$

sodium chloride were injected and the needle withdrawn. By means of a Geiger-Mueller counter tube placed 6-10 cm from the lateral aspect of the knee, the amount of gamma radiation from the knee was automatically recorded on a rate meter at 2-minute intervals. The subject was not allowed any activity during the course of the experiment. All recordings were made with the leg in extension. Continuous observations were made over a 40-minute period. A total of 35 healthy adults consisting of 12 males and 23 females was studied. Two of the males and one of the females were studied on 2 different occasions. None had any preceding history of arthritis or traumatic injury to the knee. The ages of the subjects ranged from 18-40 years.

Results. This technic for the study of Na^{24} clearance from the knee joint reveals that there is a continuous decrease of Na^{24} from the synovial space which proceeds in an exponential curve. Variations from individual to individual occur only as variations in the slopes of the logarithmic curves. The logarithmic concentration of Na^{24} within the joint was plotted against time elapsed from injection of Na^{24} into the joint space. From these data one can determine percent remaining after a 30-minute period, or calculate a clearance constant from Kety's(1) formula, $K = \frac{(\log C_1 - \log C_2)}{0.4343\ (t_2 - t_1)}$, where C_1 and C_2 equal counts per minute at times t_1 and t_2, respectively.

In Table I the results of calculation of mean absorption rate are recorded. It will be observed that the men had a mean of 48.2 $\pm$ 2.3% remaining after 30 minutes, whereas the women had a mean of 40.8 $\pm$ 1.8% remaining after 30 minutes. Statistical analysis of these figures reveals that this is a significant difference.†

When the results on female subjects are related to the day of the menstrual cycle, it is observed that more rapid clearance occurs in the early stages of the cycle. The mean percent of Na^{24} remaining after 30 minutes for each of the 3 periods was 0-9 days: 35.9 $\pm$ 3.3%, 10-19 days: 42.1 $\pm$ 2.8%, 20-28 days: 43.8 $\pm$ 3.3% (Table I).

Because of the high degree of variability among the means of the group of women in different stages of the menstrual cycle, one cannot state that there is statistical evidence of differences. However, on the basis of the means alone there is some evidence of a trend in the rate of clearance of Na^{24} during the menstrual period. These data suggest that most rapid clearance of Na^{24} is present during the early phase of the menstrual cycle.

The possibility exists that these variations might be attributable to a difference in absorbing surface area within the knee joint. On the assumption that an estimation of body surface area may be proportional to the synovial surface area, a comparison of body surface area with Na^{24} clearance was made. No correlation is found.

A comparison of clearance of Na^{24} from the synovial space with the rate of removal from muscle and subcutaneous tissue reveals that removal from synovial space is the least rapid (Table II). From the data obtained by Forbes(2) and Kety(1) it is evident that Na^{24} injected subcutaneously is removed most rapidly, while intramuscular injection of Na^{24} shows an intermediate clearance rate between subcutaneous tissue and joint space.

Kety(1) has shown that the rate of transport of Na^{24} from muscle is dependent on a constant circulation of blood. Similar ob-

TABLE II. Comparison of Clearance of Na^{24} from Various Body Sites.

Sites of administration	Mean K value	Range of K	No. of observations
Subcutaneous	.104	.040–.150	7(2)
Intramuscular	.050	.033–.066	8(1)
Synovial space of knee	.029	.014–.041	38

† "Students" t = 2.5 which has a probability of about .03 if there is no difference between the means.

servations were made in the present investigation on 2 subjects who developed a mild circulatory collapse immediately after injection of Na^{24} into the synovial space. Continuous recordings of Na^{24} concentration in the knee were made. During the period of faintness and hypotension the Na^{24} absorption was slow. Upon recovery the absorption of Na^{24} was more rapid. Thus the removal of sodium from the knee joint is dependent on both membrane permeability and circulation.

Discussion. The implication of these observed variations in Na^{24} clearance from the joint space of the knee is not clear. The possible difference attributable to the stage of the menstrual cycle at which the experiment is carried out suggest, however, an hormonal control of synovial permeability to Na^{24}. The experimental work done in rabbits by Seifter (3) supports an hormonal explanation for the variations of synovial permeability found in this investigation. When phenolsulphonthalein was injected into the rabbit synovial space, the clearance was accelerated by parenteral administration of desoxycorticosterone but retarded if cortisone was administered.

Paul and coworkers(4), however, have failed to find any effect of cortisone and ACTH on synovial permeability. Utilizing the same methods as described by Seifter, they found no significant alteration of synovial membrane permeability unless cortisone was injected into the joint. The use of Na^{24} may obviate inherent difficulties of previous methods described for the study of electrolyte exchange across the synovial membrane(5) and may serve as a useful technic for the study of the influence of endocrine substances on joint physiology.

Summary. 1. By means of injection of Na^{24} into the knee joint, the clearance of Na^{24} through the synovial membrane has been studied. 2. A significantly more rapid clearance of Na^{24} from the knee joint occurs in women than in men. There is suggestive evidence that the accelerated Na^{24} clearance in women depends upon an hormonal influence associated with the menstrual cycle. 3. Removal of Na^{24} from the joint space is less rapid than it is from subcutaneous or muscle tissue.

Grateful acknowledgement is made to A. M. Dutton, for assistance in statistical analysis of the data, and to Herbert Mermagen, for his valuable assistance in instrumentation techniques.

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Received June 19, 1952. P.S.E.B.M., 1952, v80.

Effects of Liver Fractions and Vitamin B_{12} on Body and Organ Weights of Thyroid-Fed Rats. (19722)

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The recent data of Ershoff(1,2), Betheil *et al.*(3,4), and Emerson and Folkers(5,6) indicate that liver contains an "anti-thyrotoxic factor" which counteracts the growth retardation of young rats when fed a purified diet containing thyroid. According to Ershoff (7-10), this factor is present mainly in the water insoluble fraction of liver (liver residue) and is reported to be distinct from vit. B_{12}.

This communication confirms these reports and includes new data on the effects of thyroid, defatted liver residue, liver concentrate N.F., and vit. B_{12} on some organ weights of immature rats.

TABLE I. Basal Diet Composition.

Components	g/1000 g
Casein*	220
Sucrose	715
Salts IV†	45
Cod liver oil	20

Micronutrients in mg/kilo: Thiamin, 10; riboflavin, 20; pyridoxine, 10; d-Ca pantothenate, 100; nicotinamide, 100; inositol, 50; para amino benzoic acid, 300; choline, 1000; biotin, .5; pteroylglutamic acid, 2; α-tocopherol, 142; 2-methyl 1,4-naphthoquinone, 142.

* Vitamin free casein, Nutritional Biochemicals Corp., Cleveland, Ohio.

† Hegsted *et al.*(11).

Procedure. Male, weanling albino rats weighing 35 to 45 g were kept in suspended, mesh-bottomed cages and weighed weekly. Food and water were given *ad libitum.*

The composition of the basal diet is given in Table I.

Supplements were added as per cent of the basal diet unless otherwise indicated. The following supplements were used:

Thyroid U.S.P. (*Wilson*).

Defatted liver residue (*Wilson*), insoluble fraction from pork liver after removal of water soluble substances. Dried *in vacuo,* extracted with benzol and the solvent removed *in vacuo.*

Liver concentrate N.F. (*Wilson*), water soluble fraction from pork liver.

Vitamin B_{12}, Cobione Merck.

Results. Our results are shown in Table II. In our colony 0.25% thyroid depresses growth sufficiently and allows many of the animals to live for 28 days. Addition of 10% defatted liver residue counteracts the effect of thyroid on growth and permits most of the animals to survive the experimental period. Liver concentrate N.F. or vit. B_{12} is without significant effect. This confirms the published data of Ershoff(10).

Our data indicate that the adrenal and spleen are hypertrophied while the thymus is atrophied as a result of thyroid feeding. The effect on the adrenal has been reported by Ershoff(12), and by Leathem and Howell (13). Our data show that the addition of defatted liver residue tends to restore adrenal weight to normal but does not change the enlarged spleen due to thyroid feeding. Liver concentrate and vit. B_{12} restore the spleen

TABLE II. Effect of Liver Residue, Liver Concentrate and Vitamin B_{12} on Body and Organ Weights of Thyroid Fed Rats.*
(Each experiment continued for 28 days.)

Dietary supplement	Thyroid, %	No. of rats		No. of exp.	Gain in body wt, g (mean ± S.E.)†	Organ wt, mg/100 g body wt (mean ± S.E.)†		
		Start	End			Adrenal	Thymus	Spleen
None	None	51	50	7	105.3 ± 6	17.23 ± .8	285.1 ± 19.7	545.5 ± 76
None	.25	51	31	7	69.7 ± 1.8	43.86 ± 3.58	211.1 ± 24	775 ± 83.6
Defatted liver residue, 10%	.25	51	44	7	98.1 ± 4	24.29 ± 1.81	272.3 ± 17.7	742.8 ± 79
Liver concentrate N.F., 4%	.25	32	25	4	77 ± 1.68	35.5 ± 2.22	272.5 25	549 ± 30.6
Vitamin B_{12}, 30 μg/kg	.25	22	9	3	74 ± 2.89	42.3 ± 6.5	152 ± 41.6	470 ± 52.5

* In one experiment 12.5 mg of lyxoflavin per kg of diet was without effect on weight loss due to thyroid feeding. We are indebted to Dr. Karl Folkers of Merck and Co. for crystalline lyxoflavin employed.

† Stand. error $= \sqrt{\frac{d^2}{(n)\ (n-1)}}$ where "d" is the deviation from the mean and "n" is the number of experiments.

weight to normal. Neither vit. B_{12} nor liver concentrate has an effect on the enlarged adrenal.

Atrophy of the thymus due to thyroid feeding on a soy bean diet has been previously reported from this laboratory(14). It has also been produced by Leathem and Howell (13). In our experiments the addition of defatted liver residue restores the thymus weight to normal. Liver concentrate has a similar effect on the thymus, whereas vit. B_{12} is without effect.

Discussion. Our data, using a large number of animals, confirm the work of others that liver residue counteracts the growth retardation of young rats when fed a purified casein diet containing thyroid. The effect is not due to vit. B_{12} since the addition of 30 μg of this vitamin or 40 g of liver concentrate N.F. containing 120 μg of cyanocobalamin activity to a kilo of food is without effect. Ten per cent of defatted liver residue which contributes only 20 μg of cyanocobalamin activity per kilo of food is effective.

Liver concentrate is as effective as defatted liver residue in restoring the atrophied thymus to normal. In these experiments vit. B_{12} is ineffective, which is contrary to results obtained in this laboratory using a soy bean diet (14). A difference in basal diet may account for these results.

The effect of liver concentrate on the spleen may be due to its B_{12} content since this vitamin restores spleen size to normal in our experiments.

Experiments are in progress comparing defatted liver residue with other materials as a source of "antithyrotoxic factor." Emerson and Folkers(6) reported antithyrotoxic activity for lyxoflavin on a thyroid-soy bean diet. We have fed 12.5 mg of lyxoflavin per kg of diet and observed no effect on weight loss due to thyroid feeding. This disagreement between laboratories may be due to a difference in basal diets employed.

Summary. Addition of thyroid to a casein-sucrose diet retards growth, causes hypertrophy of adrenal and spleen and atrophy of the thymus. Feeding 10% of defatted liver residue counteracts growth retardation and restores the adrenal and thymus weights to normal. It is without effect on the spleen. Liver concentrate N.F. has very little effect on growth or on the adrenal, but does tend to restore the thymus and spleen to normal. Vit. B_{12} is without effect except in respect to the spleen where its prevents hypertrophy of this organ due to thyroid feeding. Lyxoflavin was found to have no antithyrotoxic effect on our diet.

The authors wish to express their appreciation to Dr. David Klein for his interest and support and to Joan Michalski for technical assistance.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Effect of Sub-lethal Total Body X-radiation on Susceptibility of Mice to *Clostridium septicum* Toxin.* (19723)

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Numerous workers have investigated the effect of exposure to large doses of X-radiation on the susceptibility of animals to experimental infection with bacterial(1,2), viral (3,4), and rickettsial(5) agents. It has been found that irradiated animals generally, but not always, developed clinical symptoms or succumbed, when given doses of these viable agents which were too small to lead to the development of clinical disease in non-irradiated control animals. The enhanced susceptibility of irradiated animals has been attributed to the deleterious effects of exposure to large doses of X-rays in bringing about 1) lowered peripheral granular leucocytes and lymphocyte counts, 2) suppression of bone marrow and lymphatic tissue elements, 3) inhibition of antibody production, 4) altered activity of the fixed and wandering phagocytic cells, and 5) altered local tissue immunity. For pertinent reviews, the reader is referred to the papers of Taliaferro and Taliaferro(6), Craddock and Lawrence(7), and Shields Warren(8). The impediment of lymphatic blockade, and of the screening action of liver and spleen has also been held responsible(9).

The use of agents which multiply in the host before and during the period of clinical disease entails a series of complicating factors which make it difficult to deduce from experimental results the possible causes of the enhanced susceptibility of irradiated animals. A bacterial toxin appeared to be a somewhat less complicated tool for the study of susceptibility changes in irradiated animals. The toxin of *Clostridium septicum* was selected for this study because of the practical interest inherent in the problem of gas gangrene in man or animals previously exposed to ionizing radiation. This agent also offered the advantage of relatively rapid action(10), a factor of some importance in the study of the causes of radiation-induced increases in susceptibility in view of the findings that the rate of recovery from radiation damage of different tissues and functions proceeds at dissimilar rates, and that the time lapse between irradiation and infection is a critical factor in general(11).

Materials and methods. Female albino mice 5 to 6 weeks of age, from a single source (Rockland Farms) were used throughout these experiments. The method employed for exposure to X-rays was described in detail elsewhere(12). Briefly, the radiation factors were as follows: 200 KVP, 20 ma, tsd 75 cm, filter 0.25 mm Cu and 1 mm Al, HVL 0.75 mm Cu, output approximately 21 r per minute measured in air.

The authors are indebted to Dr. H. A. Dettwiler of Eli Lilly and Co., for a supply of dried *Clostridium septicum* toxin ("Vibrion, L-8392-C"), of specific horse-antitoxin ("Vibrion, No. 433515"), and a culture of *C. septicum* (No. 585). While the same lot of antitoxin was used in all experiments, both the dried toxin and freshly prepared toxic filtrates were employed.

In preparing toxin, a medium consisting of proteose No. 3 broth (proteose peptone No. 3, Difco, 20 g; NaCl, 5 g; Na_2HPO_4, 5 g; distilled water, 1,000 ml) was poured over a shallow layer of defatted horse meat in an Erlenmeyer flask and was autoclaved for one hour at 20 lb pressure, cooled rapidly, and inoculated with 5 ml of a 24-hour broth culture of *C. septicum* for each liter of medium. Sufficient sterile 40% glucose solution was added to raise the concentration of glucose in the medium to 0.2% at the beginning of the incubation period, to 1% after 18 hours of incubation, and finally to 2% after 24 to 30 hours of incubation.‡ After 24 hours of incubation

* This investigation has been supported, in part, by the U. S. Atomic Energy Commission.

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‡ The gradual increase in glucose concentration was suggested in a personal communication from Dr. H. A. Dettwiler.

TABLE I.

A. LD_{50} of *Clostridium septicum* toxin for irradiated and non-irradiated control mice.

	Mice, dead/total, after inoculation of indicated amount of toxin (ml)												
	.03	.04	.05	.06	.07	.08	.09	.10	.12	.14	.16	.18	.20
X-rayed*	0/3	1/3	1/3	1/3	2/3	2/3	1/3	2/3	3/3	3/3	3/3	3/3	3/3
Control	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	2/3	2/3	3/3

LD_{50}X (X-rayed): .07 ml
LD_{50}N (Control): .15 ml

B. PD_{50} of specific antitoxin in mice injected with .20 ml of toxin.

	Mice, dead/total, after injection of antitoxin Reciprocal of antitoxin dilution†								
	20	40	80	160	320	640	1280	2560	Normal serum‡
X-rayed*	1/6	1/6	0/6	3/6	3/6	4/6	6/6	6/6	5/5
Control	0/6	0/6	1/6	0/6	0/6	3/6	4/6	4/6	6/6

PD_{50}X (X-rayed): 1.97×10^{-3} ml
PD_{50}N (Control): 5.71×10^{-4} ml

* 350 r total body X-radiation 6 days before administration of antitoxin, and 7 days before injection of toxin, respectively.

† Antitoxin diluted in normal horse serum 1:20. .5 ml aliquots were given intramuscularly.

‡ Normal horse serum 1:20.

at 37°C samples were removed at approximately 2-hour intervals and were titrated for their hemolytic activity against sheep erythrocytes(13). The toxin was harvested by centrifugation and was filtered through a Seitz sterilizing pad when no further increases in the hemolytic activity of the culture were noted. The sterile filtrates were stored at 4°C for no longer than one week and showed little deterioration during this storage period.

Solutions of the dry toxin, as well as dilutions of the filtrates were prepared in proteose No. 3 broth just prior to their use in animal inoculations. Dilutions of antitoxin were prepared in a 2% solution of normal horse serum in physiological saline solution. The toxin was given routinely by the intraperitoneal route (0.5 ml of the various dilutions), while antitoxin was given intramuscularly (0.5 ml).

Since most of the animals which received one or more lethal doses of toxin died within 24 hours after injection, and because generally no deaths occurred later than 48 hours after injection of the toxin, all experiments were terminated 4 days after toxin had been given to the animals.

In titrations of LD_{50} dose of toxin and PD_{50}§ dose of antitoxin, 3 to 4 mice were used for each dose in those experiments in which the dose-increment was kept very small; in other experiments 6 to 8 animals were used for each dose. Both LD_{50} and PD_{50} values were calculated according to the method of Reed and Muench(14).

Experimental. Shown in Table I are a protocol and representative data of LD_{50} and PD_{50} titrations of *C. septicum* toxin and antitoxin in irradiated and in non-irradiated control mice. It may be seen that the LD_{50} dose of this particular lot of toxin was approximately 0.07 ml for mice which had been exposed to 350 r total body X-radiation 7 days before the test, while the LD_{50} for the control animals was approximately 0.15 ml. Additional results of LD_{50} titrations in mice irradiated with 250 or 350 r one week before the test have been summarized in Table II, together with the results of titrations in non-irradiated control mice. It appears that under the conditions of these experiments irradiated mice consistently succumbed to smaller amounts of toxin than did the non-irradiated controls. The ratios of LD_{50} doses for control and irradiated animals ranged from 1.2 to 2.3. The higher ratios were charac-

§ The term PD_{50} (protective dose $_{50}$) is used to denote the smallest amount of anti-toxin which protects 50% of the animals exposed to an arbitrary challenge dose of toxin.

TABLE II. LD_{50} and PD_{50} Doses of *C. septicum* Toxin and Antitoxin in X-irradiated and in Control Mice.

Toxin preparation	LD_{50} for Normal mice	LD_{50} for Irradiated mice	Challenge dose of toxin	PD_{50} for normal mice, ml	PD_{50} for irradiated mice, Observed, ml	PD_{50} for irradiated mice, Expected,* ml	% difference†
1	.66 mg	.29 mg‡	1 mg	3.71×10^{-3}	8.25×10^{-3}¶	7.75×10^{-3}	+ 6.6
2	.15 ml	.07 ml ‡	.20 ml	5.71×10^{-4}	1.97×10^{-3}¶	1.48×10^{-3}	+24.8
3	.21 "	.17 " §	.25 "	5.35×10^{-4}	8.83×10^{-4}‖	8.56×10^{-4}	+ 3.1
3	.21 "	.17 " §	.40 "	2.08×10^{-3}	2.17×10^{-3}‖	2.52×10^{-3}	−16

* Expected PD_{50} for irradiated mice $= PD_{50}$ for normal mice $\times \frac{\text{challenge dose} - LD_{50} \text{ for irrad. mice}}{\text{challenge dose} - LD_{50} \text{ for normal mice}}$.

† % difference $= \frac{(\text{observed} - \text{expected})\ PD_{50} \text{ for irradiated mice}}{\text{observed } PD_{50} \text{ for irradiated mice}} \times 100.$

‡ Mice challenged 7 days after exposure of the entire body to 350 r.

§ Mice challenged 7 days after exposure of the entire body to 250 r.

¶ 350 r total body x-radiation 6 days before administration of antitoxin, and 7 days before inj of toxin, respectively.

‖ 250 r total body x-radiation 6 days before administration of antitoxin, and 7 days before inj of toxin, respectively.

teristically found when LD_{50} doses for mice exposed to 350 r were compared with similar values for the normal animals.

It may also be seen from the results presented in Table I that more antitoxin was required to protect irradiated mice against an arbitrary lethal dose of toxin than was needed for the protection of control mice which received the same amount of toxin. In this particular experiment the PD_{50} dose of antitoxin for irradiated mice was about 2.9 times greater than the PD_{50} dose for control mice. In similar titrations, summarized in Table II, the ratio of PD_{50} doses for irradiated and for control mice varied from 1.1 to 2.2.

These results indicated that exposure of the animals to X-radiation resulted in injury which increased the susceptibility of the animals to the toxin of *C. septicum,* and that more antitoxin had to be given to irradiated mice than to non-irradiated mice in order to protect the animals from death when an arbitrary lethal dose of toxin was injected.

Discussion. The finding of increased susceptibility to the toxin of *C. septicum* among mice which had previously been exposed to sub-lethal X-radiation is consistent with the observation that previously irradiated animals generally appear to be more susceptible to a great number of different pathogenic agents. That the difference in LD_{50} doses of this toxin for irradiated mice and for non-irradiated control animals was not of the order of magnitude encountered in similar experiments with certain viable bacterial agents (1,2) may indicate that anti-bacterial defense factors are more severely damaged by X-radiation than are antitoxic defenses.

The fact that more antitoxin was required for the protection of previously irradiated mice might have been the result of radiation-induced damage to the factors and mechanisms responsible for the distribution and disposition of parenterally introduced foreign proteins (antitoxin in this case). On the other hand this observation might merely have reflected the greater susceptibility of irradiated mice to the lethal action of toxin. It could be expected that irradiated mice would require more antitoxin for their protection because in their case more of the toxin contained in the challenge dose needed to be neutralized.

It may be seen from Table II that the amount of antitoxin required to protect 50% of non-irradiated control mice against 0.40 ml of a particular preparation of toxin (preparation 3) was 2.08×10^{-3} ml while 5.35×10^{-4} ml of antitoxin sufficed to protect 50% of a similar group of mice against 0.25 ml of the same toxin. Since the PD_{50} dose of antitoxin may be considered to be the smallest amount which neutralizes, *in vivo,* all but one LD_{50} dose of the arbitrary challenging dose of toxin, and since the LD_{50} dose was found to be 0.21 ml of this preparation of toxin, one may cal-

culate that 5.35×10^{-4} ml of antitoxin were required to neutralize 0.25-0.21, or 0.04 ml of toxin. It may then be predicted that mice challenged with the larger dose of toxin (0.40 ml) would require $5.35 \times 10^{-4} \times \frac{0.40-0.21}{0.25-0.21}$, or 2.54×10^{-3} ml of antitoxin as PD_{50} dose. This expected amount of antitoxin approached closely the experimentally observed value of 2.08×10^{-3} ml (a difference of 22%), and therefore appeared to warrant similar calculations of expected PD_{50} values in irradiated mice. These calculations were based on the assumption that irradiated mice, tested under the conditions of these experiments, were as capable of utilizing passively administered antibody as were the corresponding non-irradiated control mice.

It may be seen from Table II that the predicted PD_{50} values differed by no more than 25% from the experimentally observed values. In view of the errors inherent in the calculations of LD_{50} and PD_{50} doses, even the greatest difference between expected and observed values encountered in these experiments appeared to lack significance. The data therefore appear to support the hypothesis that irradiated and non-irradiated control mice do not differ from each other in their ability to utilize antibody.

Though other results obtained in this laboratory(2) indicated that the difference in LD_{50} doses of tetanus toxin for irradiated and non-irradiated mice is, like that of *C. septicum* toxin, only 2- to 3-fold, further data would be required to establish the validity of such a generalization for susceptibility to the action of bacterial toxins. It appears possible, however, that X-radiation may not necessarily result in selective severe damage to antibacterial defenses, but that exposure to sublethal X-ray may rather lead to modifications of host tissues or enzyme systems which might result in the production of a more favorable environment for the propagation and spread of viable agents.

The efficacy of passive serum prophylaxis against *C. septicum* toxin in animals which had received large, though sublethal doses of X-rays is of obvious practical value if these findings were to hold equally true for exposure to other ionizing radiations, and to animals other than mice. It should be noted that the antitoxin was given 6 days after exposure of the mice to X-radiation, and that at this particular post-irradiation time increases in capillary permeability were presumably quite pronounced(15). It is interesting to observe that, in spite of the presumptive "leakage" of antibody from the circulatory system(16), the animals could be effectively protected with amounts of antitoxin which were not greatly in excess of those required for the protection of non-irradiated control mice, and that the difference in the PD_{50} doses for irradiated and control mice could well have been due to the fact that, since irradiated mice succumbed to smaller amounts of (not neutralized) toxin than the controls, a greater proportion of the challenge dose of toxin needed to be neutralized in the case of irradiated animals.

Summary and conclusions. Total body exposure to 250 and 350 r reduced the resistance of mice so that animals injected 7 days after irradiation succumbed to approximately one-half the amount of *C. septicum* toxin which was required to kill non-irradiated control mice. Antitoxin given intramuscularly 6 days after irradiation, and one day before challenge with toxin, protected the life of both irradiated and control mice. Irradiated mice required from 1.1 to 2.2 times more antitoxin for their protection than did control animals. The increased antibody requirement of irradiated mice could be explained by the enhanced susceptibility to toxin of these animals. The results indicated that exposure to severe doses of X-radiation, in spite of its effects on capillary permeability, apparently fails to significantly raise the minimum amount of antibody required for effective antitoxic prophylaxis.

The authors gratefully acknowledge the cooperation of Dr. Hugh M. Wilson of the Mallinckrodt Institute of Radiology in making available the facilities for irradiation of animals.

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Received July 21, 1952. P.S.E.B.M., 1952, v80.

Effect of Administration of Amino Acids on Circulating Eosinophils and Lymphocytes in Normal and Adrenalectomized Rats. (19724)

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It has been known for some time that glutamic acid is the only amino acid that increases the oxygen consumption when added to nervous tissue *in vitro*(1,2). Weil-Malherbe(3) has made the claim that the therapeutic effect of the administration of glutamic acid in certain diseases of the central nervous system as reported by some investigators(4) was due to the liberation of adrenalin and not to the unique behavior of this amino acid on nervous tissue. (The controversy regarding the therapeutic efficacy of glutamic acid is outside the scope of this paper.) Since it has been shown that adrenalin will produce an eosinopenia and lymphopenia in animal and man(5-8), it was thought that similar effects should be produced by the administration of glutamic acid if the claim of Weil-Malherbe were correct.

A study was made of the effect of the oral administration of various amino acids and water or 0.5% saline on the blood picture of a group of white rats before and after adrenalectomy.

Procedure and methods. Each of a group of 12 male rats weighing approximately 200 g at start of the experiment was given 5 ml of a 5% solution of l-glutamic and l-aspartic acid as their mono-sodium salts, glycine, dl-α alanine and water by stomach tube with a rest period of several days between the administration of each of these constituents. The rats were not fasted overnight. The food was removed from the cages at 9 a.m. before the experiments were started. Water was allowed *ad lib.* The tail of the rat was warmed in water and blood was obtained by cutting the lateral vein of the tail near its distal end with a razor blade. Blood counts were made immediately before and 4 hours after the administration of the test material. A complete series with the various substances was carried out on each rat and the blood eosinophil count was determined by the method of Randolph (9). The complete series was then repeated on the same rats and total white, differential, and total lymphocyte counts were made. After completion of the 2 series the same rats were adrenalectomized and the same tests were repeated. Thus each rat was subjected to 20 experimental tests. Three of the 12 rats were lost as a result of the operation.

The adrenalectomized rats were given 5% glucose in physiological saline solution for drinking and the amino acids were dissolved in 0.5% saline instead of water. The rats were treated, otherwise, in the same manner as before adrenalectomy except that during the

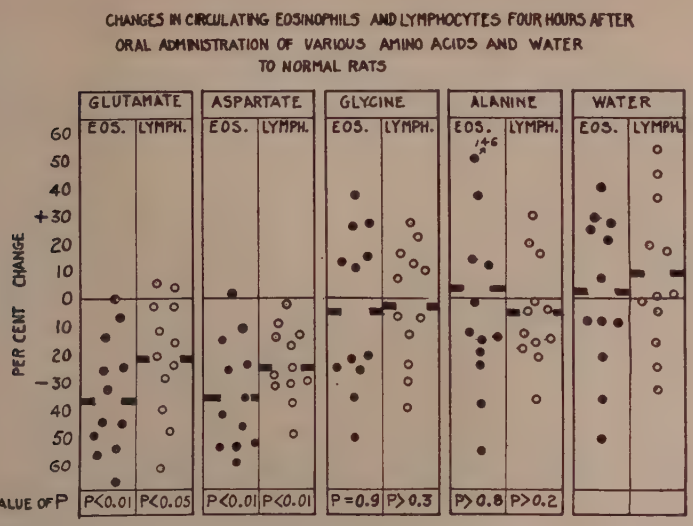

FIG. 1. Each point represents result of a test on each of 12 rats. Black dots represent % change in circulating eosinophils from the initial level, and open circles that of % change in level of circulating lymphocytes. Heavy horizontal bars mark the mean values. P values greater than .05 were not considered statistically significant.

4-hour experimental period the drinking water was replaced by 0.5% saline. The 9 adrenalectomized rats appeared well throughout the period of the experiments. In only one animal a very small adrenal rest was found on autopsy.

The effect of the administration of each amino acid on the per cent change in the absolute number of circulating eosinophils and lymphocytes was compared with that of water or saline as control for each rat. The data were analyzed statistically for significance.

Observations and results. Changes in the level of blood eosinophils. The administration of glutamic and aspartic acid resulted in a depression of the absolute eosinophil count in all of the normal animals but one in each group. The fall ranged from 7% to 67% in the former and from 11% to 60% in the latter with a mean of 36% and 35%, respectively. The per cent change in the absolute eosinophil count was compared for each rat against its water control and the statistical significance of the difference gave a value of $P < 0.01$ and $P < 0.05$, respectively. The administration of water, glycine and alanine gave marked plus and minus changes in the absolute eosinophil count (Fig. 1) and the changes due to these amino acids were not statistically significant.

Following adrenalectomy the administration of glutamic and aspartic acid was followed by a fall in the absolute eosinophil count except for one animal in each group which showed no change and one animal in the latter group which had a rise of 5%. The mean of the per cent change was minus 30.1% and minus 18%, respectively. These means were somewhat smaller than those obtained prior to adrenalectomy. The statistical significance of the changes compared against saline gave $P < 0.01$ (Fig. 2). The administration of 0.5% saline, glycine and alanine to the adrenalectomized rats again showed marked plus and minus changes in the eosinophil count and the effects of these amino acids were not statistically significant (Fig. 2). Thus the administration only of glutamic and aspartic acid produced a statistically significant change in the eosinophil count both before and after adrenalectomy although it was somewhat greater prior to adrenalectomy especially in the case of aspartic acid.

Changes in the blood level of lymphocytes. The administration of glutamic and aspartic acid was accompanied by a depression of the

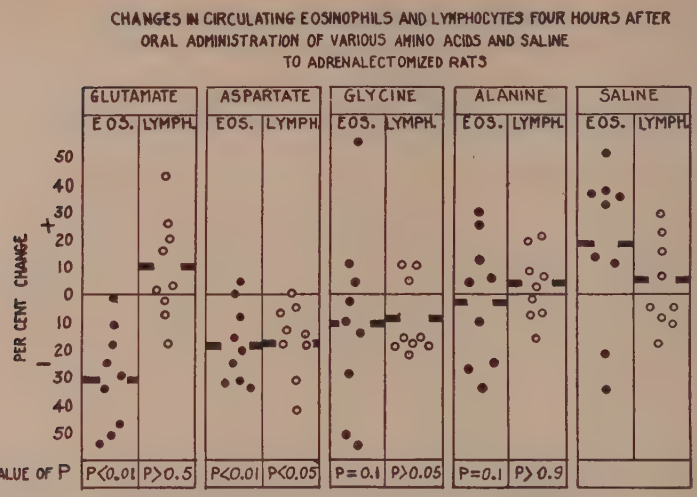

FIG. 2. Legend is the same as in Fig. 1 except that only 9 animals remained alive after adrenalectomy.

absolute lymphocyte count in all of the normal animals except in 2 of the former in which there was a slight rise. The mean of the per cent change was minus 20.7% and 23.8%, respectively. The per cent change for each rat compared with its water control was statistically significant with $P < 0.05$ and $P < 0.01$, respectively, for glutamic and aspartic acid.

The administration of water, glycine and alanine was accompanied by marked plus and minus changes in the absolute lymphocyte count (Fig. 1) which were not statistically significant.

Following adrenalectomy the administration of aspartic acid was accompanied by a depression of the lymphocytes in all the animals with a mean value of the per cent change equal to 17.7% as compared to 23.8% before adrenalectomy. The P value calculated as above against saline was $P < 0.05$ (statistically significant). Glutamic acid administration, however, was followed by marked plus and minus changes in the lymphocytes with a mean value of the per cent change equal to plus 0.9% and was not significant. This was in marked contrast to the effect on the eosinophils. The changes following glycine and alanine were not statistically significant (Fig. 2).

Discussion. The finding that the oral administration of sodium glutamate to normal rats produces a statistically significant depression of the blood eosinophil level, as compared to the effect of water, would be in harmony with the view of Weil-Malherbe(2) that glutamic acid stimulates adrenalin secretion. However, since a similar effect is produced in the same animals after adrenalectomy, it is obvious that some mechanism, other than adrenalin secretion and stimulation of the pituitary-adrenocortical system, accounts in large part for the observed effect. Were one to use the change in the lymphocyte count as an indicator, the absence of a statistically significant fall in the lymphocytes in the adrenalectomized rats would lend support to the view of Weil-Malherbe. (Studies on adrenal demedullated rats are being planned for further elucidation). However, since the administration of aspartic acid produced a statistically significant depression in the level of both circulating eosinophils and lymphocytes in the normal and adrenalectomized state, it is apparent that other mechanisms, besides adrenalin secretion and stimulation of the pituitary adrenocortical system, must play a role in affecting the level of these blood constituents. It is possible, however, that the lesser effects produced by glutamic and aspartic acid in the adrenalectomized animals indicate that part of their effects are produced through the

adrenals and perhaps via adrenalin secretion. It is hoped that further studies on adrenal demedullated rats may throw some light on this point.

The mechanisms which account for the difference between the mono and dicarboxylic amino acids and between glutamic and aspartic acid are obscure at present. It may be of interest to note that Awapara(10) has reported that the adrenals appear to play some significant role in the metabolism of the dicarboxylic amino acids.

Summary and conclusions. 1. A study was made to determine whether the oral administration of glutamic acid produced an increased secretion of adrenalin by employing the changes in the blood eosinophil and lymphocyte level as indicators. 2. Glutamic acid, aspartic acid, glycine, alanine, and water or saline were administered to a group of white rats before and after adrenalectomy and the effects on the levels of the blood eosinophils and lymphocytes were determined. 3. Only the dicarboxylic amino acids produced a statistically significant depression of the eosinophils in the normal and adrenalectomized animals. However, in the latter animals the lymphocytes also were depressed by aspartic acid but not by glutamic acid. 4. The results obtained in adrenalectomized rats showed that the levels of the blood eosinophils and lymphocytes could be influenced by other mechanisms than the pituitary-adrenal system. 5. The possible significance of the small difference observed between normal and adrenalectomized rats regarding eosinophil changes is discussed.

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Received July 8, 1952. P.S.E.B.M., 1952, v80.

Effects of Adrenal Ligation and Adrenergic Blockade on Pressor Responses to Acetaldehyde in Dogs.* (19725)

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The use of tetraethylthiuram disulfide (Antabuse) in the treatment of chronic alcoholism has stimulated interest in the mechanisms involved in the Antabuse-alcohol reaction. At the present time, it is believed that the acetaldehyde formed from ingested alcohol produces the symptoms of the Antabuse-alcohol reaction in which hypotension is characteristic(1). Nelson(2) has shown that acetaldehyde causes a transient pressor response in dogs after both carotid arteries are clamped, the vagus nerves sectioned, the adrenal vessels ligated, and after ganglionic blockade induced by nicotine. Reversal of the pressor response by the adrenergic blocking drug 2-(N, p-tolyl-N [m′ oxyphenyl] aminomethyl) imidazoline (C-7337) has been demonstrated by Christensen(3) to occur in the intact anesthetized dog.

These observations indicate that acetaldehyde is a peripherally acting drug. The present experiment was undertaken to confirm 2 aspects of the evidence: 1) that the pressor response is not due to the release of epinephrine from the adrenal medulla; 2) that the pressor response is reversed by an adrenergic

* The materials required for this work were made available through the kindness of Professor George L. Maison, Dept. of Pharmacology, Boston Univ. School of Medicine.

TABLE I. Effect of Adrenal Ligation and SY-28 on Blood Pressure Responses to Epinephrine and Acetaldehyde in Dogs.

Procedure	Mean dose & S.D. l-epinephrine tartrate, γ/kg	Mean pressor response & S.D., mm Hg	Mean dose & S.D. acetaldehyde, mg/kg	Mean pressor response & S.D., mm Hg
Before adrenal ligation	4.67 ± 2.76	70 ± 41.8	9.37 ± 1.76	45.7 ± 15.9
After adrenal ligation	4.07 ± 3.31*	57.4 ± 41.8*	9.37 ± 4.96	24 ± 7.73
		Mean depressor response & S.D., mm Hg		Mean depressor response & S.D., mm Hg
After† adrenal ligation & after SY-28	1.1 ± .86	28.5 ± 19.2	8.25 ± 3.46	22.5 ± 5.9

* Seven observations.
† Four experiments; all other data represent 8 experiments.

blocking agent after ligation of the adrenal vessels.

Procedure. In 4 male and 4 female dogs anesthetized with pentobarbital sodium (approximately 30 mg/kg) the right common carotid artery and right femoral vein were cannulated. Blood pressure was recorded by a mercury manometer. Prior to and following a one-stage bilateral ligation of the adrenal vessels, acetaldehyde (Mallinckrodt), and l-epinephrine tartrate were given intravenously as *statim* injections. After the second series of injections, the adrenergic blocking agent N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine • HBr (SY-28) was administered intravenously over a period of 10 minutes to 2 male and 2 female dogs at a dosage of 1 mg/kg. Fifteen minutes later presence of adrenergic blockade was tested by injecting l-epinephrine tartrate. When blockade was complete, as shown by epinephrine reversal, acetaldehyde was given. The completeness of the adrenal ligation was confirmed at autopsy in all animals.

Results. The results of these experiments, presented in Table I, indicate that acetaldehyde causes a pressor response in dogs before and after the adrenal vessels are ligated. Following the adrenal ligation, the pressor response is reversed by an adrenergic blocking agent.

Discussion. This experiment confirms the observation of Nelson(2) that the pressor response to acetaldehyde is not wholly due to the release of epinephrine from the adrenals. Moreover, the pressor response is reversed by SY-28 after the adrenal vessels are ligated. The fact that statim injections of acetaldehyde and its continuous administration as a volatile anesthetic(4) produce a rise in blood pressure in dogs is at variance with the present belief that acetaldehyde causes the hypotension of the Antabuse-alcohol reaction in humans.

Summary. 1. The pressor response to acetaldehyde in dogs is not wholly due to the release of epinephrine from the adrenals. 2. The pressor response to acetaldehyde is reversed by the adrenergic blocking drug SY-28 following bilateral ligation of the adrenal vessels.

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Received July 18, 1952. P.S.E.B.M., 1952, v80.

Effect of Cortisone Acetate on Growth of Strain Specific Tumors in Alien Strains of Mice. (19726)

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In a previous publication(1) experiments were described in which CF_1 mice implanted with lymphosarcoma 6-C3H-Ed (originating in C3H mice) and treated with cortisone acetate died of progressive growth of the tumor. Similar observations were made by Howes(2) who described progressive growth of adenocarcinoma EO771 and 775 (originating in C57 mice) when transplantations were made to Rockland Swiss mice treated with cortisone acetate. The present report describes further studies on the effect of cortisone acetate in promoting the growth of lymphosarcoma 6-C3H-Ed in CF_1 mice and attempts to induce progressive growth of this and other strain specific tumors in other alien strains of mice treated with cortisone acetate.

Materials and methods. CF_1 and AKM mice were obtained from Carworth Farms and ZBC mice from the surplus stock of Dr. J. J. Bittner. C3H (He), DBA-2, C57 Black, A (Lilly), and A (He) mice and all of the tumors except the Patterson lymphosarcoma, which was supplied by Dr. C. C. Stock, were obtained from the Jackson Memorial Laboratory. Routine passage of the tumors was made in mice for which the tumor is specific, (lymphosarcoma 6-C3H-Ed and adenocarcinoma C3H-BA in C3H (He), lymphatic leukemia P-1534 in DBA-2, adenocarcinoma EO771 in C57 Black and the Patterson lymphosarcoma in AKM mice). Young adult mice of both sexes were used. In most experiments, cortisone was given subcutaneously as an aqueous suspension of the acetate in 0.25 ml volumes. The animals were palpated 3 times a week to determine growth behavior of the tumor. Additional details are given in connection with the experiments.

Experimental. CF_1 mice implanted with lymphosarcoma 6-C3H-Ed taken from C3H mice develop large tumors which reach their maximum size in about 2 weeks, after which rapid regression (usually complete in 2-4 days) takes place. Such mice are immune to reimplantation of the tumor. Similar immunity develops in CF_1 mice implanted with mixtures of spleen, lymph-node and thymus tissue from C3H mice. These observations suggested that lymphosarcoma 6-C3H-Ed grows progressively in cortisone treated CF_1 mice because such treatment interferes with the normal development of immune responses. The following experiment shows that the immunity which develops following implantation of lymphoid tissues from C3H into CF_1 mice is impaired by intensive treatment with cortisone acetate. Twenty CF_1 mice were implanted by trocar with fragments of spleen, lymph-node and thymus subcutaneously on the left flank. Beginning on the same day, 10 of the mice were begun on a course of treatment with cortisone acetate. Daily doses of 40 mg/kg were given for 7 successive days. Ten of the mice remained untreated. Ten days following implantation of the lymphoid tissue, all 20 mice and an additional 10 controls were implanted by trocar in the right flank with lymphosarcoma 6-C3H-Ed. The cortisone treated and the normal control mice developed large tumors, which in due course regressed; the mice implanted with lymphoid tissue and which received no cortisone failed to develop palpable tumors. These results are shown in Exp. 1 in Table I.

Experiments were made to determine whether the immunity developed by CF_1 mice in which lymphosarcoma 6-C3H-Ed had regressed could be destroyed by cortisone acetate treatment. Groups of such immune mice were treated according to different dosage schedules with cortisone acetate. In no instance was the immunity abolished, as is shown in Exp. 2 in Table I.

The finding that intensive treatment suppresses the development of the immunity of CF_1 mice induced by implantation of tissues from C3H mice (Exp. 1) suggested that serial passage of lymphosarcoma 6-C3H-Ed through groups of CF_1 mice treated with cortisone

TABLE I. Effect of Cortisone Acetate Treatment on Immunity of CF_1 Mice to Lymphosarcoma 6-C3H-ED.

Experimental procedure	Treatment	No. of mice	Immune to lymphosarcoma 6-C3H-ED
Exp. 1			
Implanted with a mixture of spleen, lymph-node and thymus tissue fragments from C3H mice	40 mg/kg/day for 7 days beginning on day of tissue implantation.	10	0*
	No treatment	10	10
Normal CF_1	" "	10	0
Exp. 2			
Mice immune as a result of regression of previously implanted lymphosarcoma 6-C3H-ED	40 mg/kg/day for 5 successive days. Tumor implanted 3 days after last dose was given.	8	8
	Treated for 7 days. Tumor implanted on day last dose was given.	8	8
	Treated for 3 days. Tumor implanted & treatment continued for 7 additional days.	13	13
	No treatment	8	8
Normal CF_1 mice		10	0

* Tumor implanted 10 days after C3H lymphoid tissue had been implanted.

acetate could be accomplished. To test this possibility, groups of 5 CF_1 mice were implanted subcutaneously by trocar with tumor removed from C3H mice. Cortisone injections (40 mg/kg/day) were begun within 4 hours after implantation and were continued for 7 successive days. Mice bearing large, obviously progressive tumors were killed between the 14th and 21st day and their tumors used for the next passage to new groups of CF_1 mice which were treated with cortisone acetate as were their predecessors. At each passage a control group of untreated CF_1 mice was implanted with the same tumor material in order to determine whether adaptation of the tumor to CF_1 mice had occurred. Two experiments were conducted. In the first, 4 serial passages were made before all mice in the treated group suddenly died; in the second, the tumor was passed in 9 successive groups of cortisone treated mice before the experiment was discontinued. In no instance did tumor taken from cortisone treated mice and transplanted into untreated CF_1 mice grow progressively. Apparently the species specificity of this C3H tumor was not altered by repeated passage in cortisone treated CF_1 mice.

The above results show that treatment with cortisone acetate impairs the development of the immunity of CF_1 mice induced by lymphoid tissue of C3H mice and it may be inferred that the progressive growth of lymphosarcoma 6-C3H-Ed in cortisone treated mice is a result of suppression of immune response to the C3H tumor tissue. Thus the "natural immunity" of CF_1 mice to lymphosarcoma 6-C3H-Ed which is manifest by regression may be related to (or identical with) specific foreign tissue immunity. In view of this, it was considered of importance to determine whether the resistance of other alien strain mice to progressive growth of this and other strain specific tumors could be abolished by intensive treatment with cortisone acetate. Table II shows the results of implantation of a variety of tumors into pure bred (DBA-2, C57, A and AKM) random bred, (CF_1) and backcross (ZBC) mice treated with cortisone acetate, together with data showing the effect of different dosage schedules on the progressive growth of lymphosarcoma 6-C3H-Ed in

TABLE II. Effect of Cortisone Acetate Treatment* on Progressive Growth of Various Tumors in Alien Strain Mice.

Implanted	Treatment with cortisone acetate	Recipient mouse	No. of mice	Growth of tumor: Progressive	Growth of tumor: No growth or regression
Lymphosarcoma 6-C3H-Ed	Normal untreated	CF_1	57	2	55†
	Mice pretreated for 3 days prior to tumor implantation	CF_1	10	0	10†
	Treated for 3 days beginning on the day of tumor implantation	CF_1	10	0	10†
	Treated for 10 days beginning on day of implantation	CF_1	30 (7)‡	17	6§
	Treated for 7 days beginning on day of tumor implantation	CF_1	62 (6)	37	19†
	"	DBA – 2	10	0	10
	"	C57 Black	10	0	10
	"	A (Lilly)	10 (1)	0	9
	"	AKM	10 (2)	0	8
Adenocarcinoma EO 771	"	ZBC	13	9	4
	"	CF_1	10	0	10
Leukemia P-1534	"	ZBC	10	0	10
	"	CF_1	10 (1)	0	9
Patterson lymphosarcoma	"	CF_1	10	0	10
	"	A (He)	10	0	10
Adenocarcinoma C3H-BA	"	CF_1	40 (5)	0	35

* Cortisone acetate aqueous suspension 40 mg/kg/day.
† Tumors grew to large size, then regressed completely.
‡ Number in parentheses indicates death during treatment.
§ Experiments previously described(1).

CF_1 mice.

Summary. Lymphosarcoma 6-C3H-Ed implanted in CF_1 mice intensively treated with cortisone acetate grows progressively, and adenocarcinoma EO-771 also grows progressively in ZBC mice under similar conditions. Neither of these tumors nor others tested grew progressively in other alien strains of mice treated with cortisone acetate. Progressive growth of tumors in alien strains of mice under the influence of cortisone acetate treatment is restricted to certain tumors in certain strains of mice, and is therefore not an expression of a general breakdown of natural barriers to tumor transplantation.

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Received July 17, 1952. P.S.E.B.M., 1952, v80.

Resistance of C3H Mice to Lymphosarcoma 6-C3H-Ed Induced by Tissues from Mice of C3H Sublines. (19727)

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Immunity against tumors which grow progressively in strains of pure bred mice by prior injections of normal tissues from alien strains of mice has been described by MacDowell *et al.*(1), and by Rhodes and Miller(2), who studied leukemia, and by Barret *et al.*(3) using a fibrosarcoma. In experiments previously reported by us(4,5) it was shown that C3H mice,* obtained from the Jackson

* In the previous publications(4,5) these mice were referred to as Jax C3H mice. Specifically they are of the Heston subline maintained at the Jackson Memorial Laboratory and in the present paper are designated as Jax-C3H (He) mice.

Memorial Laboratory, were protected against lymphosarcoma 6-C3H-Ed by previous implantation of lymphoid tissue from a variety of random bred and pure strain mice. Spleen tissue from ZBC mice was found to be particularly active as an antigen in inducing this immunity. These studies now include experiments in which lymphoid tissue from several sublines of C3H mice were implanted in mice of other C3H sublines followed by challenge with lymphosarcoma 6-C3H-Ed, and attempts were made to broaden the findings which had been made with this tumor using similar methods and other lymphoid tumors at our disposal.

Materials and methods. Jax-C3H (He), DBA-2, C57 Black, A (Lilly), and A (He) mice were obtained from the Jackson Memorial Laboratory. ZBC mice (produced by mating mice of the A strain and Dr. Bittner's C3H (called Z-strain) to produce F_1 hybrids, and mating F_1 females with Z males) were obtained, together with a supply of C3H (called Z) mice from Dr. J. J. Bittner. C3H mice of the Andervont stock were obtained from Dr. H. B. Andervont. CF_1, CFw and AKM mice were obtained from Carworth Farms and "Manor" mice from Manor Farms. Lymphosarcoma 6-C3H-Ed, (C3H (He) mice), and lymphatic leukemia P-1534 (DBA-2 mice) were obtained from the Jackson Memorial Laboratory. The Patterson lymphosarcoma (AKM mice) was obtained from Dr. C. C. Stock, and lymphoma 2 (A (He) mice) was obtained from Dr. Emma Shelton through Dr. L. L. Law. The tumors were routinely passed in the pure strain mice designated in parentheses. Approximately 15 mg pieces of lymph nodes and of the various tumors were implanted by trocar subcutaneously on the left flank of the mice. Spleen cell suspensions were implanted subcutaneously by injection, each mouse receiving approximately 18-20 mg of moist spleen tissue in 0.25 ml of saline suspension. Seven to 10 days after these preliminary implantations, the mice were challenged subcutaneously in the right flank with approximately 5 million tumor cells suspended in 0.1 ml of saline. Similar tumor injections were made in control mice of the same strain. The mice were palpated twice weekly and observed either until death, or until it became obvious that the challenge tumor had regressed or had failed to grow, as judged by progressive tumor growth and death of the appropriate control mice. Apparently immune mice were rechallenged and observed for an additional 4 weeks before being finally classified as immune. In some instances, the mice available for study was few, but, with these exceptions, all experiments were carried out with groups of 8 mice.

Results. Implantation of lymphoid tissue from mice of certain sublines of the C3H strain results in immunity of recipient mice of other C3H sublines subsequently implanted with lymphosarcoma 6-C3H-Ed. Immunity of the mice was manifest by failure of the implanted tumor to grow at all or by regression after reaching palpable size. The results of experiments in which lymphoid tissues from various sublines of C3H mice were implanted in mice of other C3H sublines, followed by challenge with lymphosarcoma 6-C3H-Ed, are shown in Table I.

Spleen or lymph node-thymus mixtures from Bittner C3H (Z) mice regularly immunizes Jax-C3H (He) mice. Spleen from ZBC mice is also effective. Spleen from Jax-C3H (He) mice immunizes Bittner C3H (Z). Spleen from both Jax-C3H (He) and Bittner C3H (Z) mice immunizes Andervont C3H mice against lymphosarcoma 6-C3H-Ed, but spleen from Andervont mice fails to immunize either Bittner C3H (Z) or Jax-C3H (He). Spleen from ZBC does not immunize Bittner C3H (Z) mice.

Experiments were made to study the effect of various treatments of spleen cells from ZBC mice on their antigenicity in Jax-C3H (He) mice and to determine whether implantation of lymphoid tissues from alien strains would immunize ZBC mice against lymphosarcoma 6-C3H-Ed, or A (He), AKM, or DBA-2 mice against lymphoid tumors which grow progressively in these strains. Results of these experiments are shown in Table II.

It is seen in Table II that none of the tissues implanted into ZBC mice induced immunity against lymphosarcoma 6-C3H-Ed, nor did tissues from the alien strains of mice immunize DBA-2 against leukemia P-1534,

TABLE I. Immunity Produced by Lymphoid Tissues Implanted in Various Sublines of C3H Mice Against Lymphosarcoma 6-C3H-ED.

Strain and type of mouse tissue implanted	Recipient mice	No. of mice	Protected	Not protected
ZBC spleen	Jax-C3H (He)	75	73	2
Bittner C3H (Z) spleen	"	11	11	
Bittner C3H (Z) lymphnode/thymus	"	12	11	1
Andervont C3H lymphnode/thymus	"	4		4
Andervont C3H spleen	"	4		4
ZBC spleen	Bittner C3H (Z)	2		2
Jax-C3H (He) spleen	"	2	2	
Andervont C3H "	"	3		3
Jax-C3H (He) "	Andervont C3H	2	2	
Bittner C3H (Z) "	"	2	2	
Growth of tumor in controls	ZBC	300+	Progressive to death	
	Jax-C3H (He)	300+	"	
	Andervont C3H	1	"	
	Bittner C3H (Z)	4	"	

TABLE II. Attempts to Induce Immunity Against Lymphoid Tumors by Implantation of Alien Mouse Tissues.

Strain and type of mouse tissue implanted	Recipient mice	No. of mice	Tumor	Protected	Not protected
Bittner C3H (Z) spleen	ZBC	13	Lymphosarcoma (6-C3H-Ed)		13
Bittner C3H (Z) lymphnode/thymus	ZBC	8	"		8
Andervont C3H lymphnode/thymus	ZBC	4	"		4
Spleen or lymphnode from: CF_1, CFW, DBA-2, C57 Black, Manor AKM, A (Lilly), A (He) Jax-C3H (He), ZBC	ZBC	160*	"		160
"	DBA-2	160*	Leukemia—P-1534		160
Spleen from above mice	AKM	80†	Patterson lymphosarcoma		80
"	A (He)	80†	Lymphoma 2		80
ZBC spleen	Jax-C3H (He)	75	Lymphosarcoma (6-C3H-Ed)	73	2
ZBC lymphnode/thymus	"	16	"	3	13
ZBC spleen (frozen and thawed)‡	"	8	"		8
ZBC " (crushed to break cells)§	"	8	"		8
ZBC " (lyophilized)	"	8	"		8
ZBC " (boiled)	"	8	"		8
ZBC " (cells suspended in .1% formalin/saline)	"	8	"		8
ZBC spleen (fresh spleen, mice treated with cortisone acetate 40 mg/kg/day/5 days)	"	16	"		16

* Groups of 8 mice each received either spleen or lymphnode from various strains of mice.
† " " " " " " " spleen from various strains of mice.
‡ Frozen at −18°C for 2 hr, then slowly thawed at room temperature.
§ Ground with sand in saline.

AKM against the Patterson lymphosarcoma nor A (He) mice against lymphoma 2. Spleen cells of ZBC mice are much more active than lymph node/thymus mixtures in immunizing Jax-C3H-Ed mice against lymphosarcoma 6-C3H-Ed. Any treatment which ruptures the cell wall abolishes the antigenicity of ZBC spleen tissue under the conditions of these experiments, and treatment with cortisone acetate prevents the development of immunity against this tumor.

Discussion. It was previously shown that lymphoid tissue from a variety of random bred mice, and from mice of known genetic constitution would immunize Jax-C3H (He) mice against progressive growth of lymphosarcoma 6-C3H-Ed. Similar relationships are shown in the present experiments in which

lymphoid tissue from mice of several C3H sublines were implanted into mice of other sublines. Mice of the Heston, Bittner(Z), and Andervont C3H sublines, and ZBC mice, in all of which this tumor grows progressively, show different behavior in immunizability following subcutaneous implantation of spleen or lymph node and thymus tissue from other sublines of C3H mice. Tissue from Bittner C3H (Z) and Jax-C3H (He) mice reciprocally immunized against this tumor. Spleen from Andervont C3H mice did not immunize Bittner C3H (Z) mice, yet spleen from the latter, and from Jax-C3H (He) mice immunized Andervont mice. ZBC spleen failed to immunize Bittner C3H (Z) mice. Although many have been tried, none of the tissues yet implanted into ZBC mice have immunized against lymphosarcoma 6-C3H-Ed.

It is of interest that lymphoid tissue from one C3H subline should immunize mice of another subline against a tumor which grows progressively when transplanted in all C3H mice tested. It is assumed that the immunity observed has as its basis the existence of antigenic differences between the tissues of one type mouse from that of the others, and whatever these differences may be, it would appear that antigenic tissues are widely distributed, for protection of Jax-C3H mice can be accomplished not only by implantation of tissues from other C3H sublines as in the present instances, but, as was shown previously(4), spleen or lymph node and thymus tissue from random bred mice, (CF_1, CFw and Manor), and pure lines (DBA-2, C57 Black) will also induce immunity. In this connection it is important to note the apparent differences in antigenicity of spleen and lymph node thymus tissue of ZBC mice in immunizing Jax-C3H (He) mice against lymphosarcoma 6-C3H-Ed-spleen regularly induces immunity, while the lymph node thymus mixture does so infrequently.

The data suggest that genetic differences between sublines of C3H mice might be detectable by immunological tests of the type described. Such differences have been previously shown to exist between the Bittner and Andervont(6) and between the Bittner and the Strong sublines(7) by differential susceptibility of the mice to transplantable mammary carcinoma.

Implantation of intact cells appears to be a requirement for antigenicity, which is in keeping with the observations of Barrett *et al.* (3) who studied immunity produced in C strain mice injected with blood from DBA mice against a fibrosarcoma which arose in DBA mice, but which was transplantable to C mice. Development of immunity in Jax-C3H (He) mice against lymphosarcoma 6-C3H-Ed is prevented in mice treated with cortisone acetate (40 mg/kg for 5 days) beginning on the day that ZBC spleen was introduced.

In our experience, the antitumor immunity developed by implantation of lymphoid tissue from certain strains of mice can be developed only against lymphosarcoma 6-C3H-Ed. Repeated trials failed to induce immunity in DBA-2 mice against leukemia P-1534, in AKM mice against the Patterson lymphosarcoma and in A (He) mice against lymphoma 2. These results suggest a close similarity in the antigenic constitution of lymphoid tissue of certain strains of C3H and other mice(4) to that of lymphosarcoma 6-C3H-Ed.

Summary. Experiments are described in which spleen or lymph node from certain sublines of C3H mice when implanted into mice of other sublines of the C3H strain immunized them against progressive growth of subsequently implanted lymphosarcoma 6-C3H-Ed. In repeated similar trials in other strains of mice, no immunity was produced against lymphoid tumors which grow progressively in these strains.

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Received July 17, 1952. P.S.E.B.M., 1952, v80.

Immunity of C3H Mice to Lymphosarcoma 6-C3H-Ed Following Regression of the Implanted Tumor. (19728)

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Immunity in mice and rats against tumors which arose in and which grow progressively in certain pure bred lines of these animals, following atrophy of the tumor, has been observed by a number of workers. MacDowell (1) by repeated injections of small numbers of leukemia cells (which failed to induce leukemia) was able to immunize C58 mice against a transplantable leukemia specific for this strain. Gross(2,3) implanted C3H mice intradermally with small quantities of a methylcholanthrene induced sarcoma which arose in this strain, many of the tumors regressed, and mice which had borne the regressive tumors were immune to subsequent subcutaneous implantations of the same tumor. Goldfedder (4) described immunity of inbred Bagg rats to a transplantable lymphosarcoma which arose spontaneously in this strain, which was induced following implantation of fragments of the tumor which had been attenuated by x-rays. More recent mention has been made (5) of similar experiments carried out with a spontaneous mammary tumor which arose in Bittner's high cancer line of mice, in which implantation of x-rayed tumor tissue induced immunity in autogenous hosts. Stoerk and Emerson(6) induced severe riboflavin deficiency in C3H mice bearing lymphosarcoma 6-C3H-Ed, the tumors regressed and those mice which survived the vitamin deficiency were found to be immune to subsequent implantation of the tumor. Lewis *et al.*(7) by use of a variety of technics were successful in impairing the blood supply of transplantable sarcoma which arose, and were progressive upon transplantation, in pure bred rats of the Lewis and King A strains. The treatments caused atrophy of the tumors which resulted in immunity of the rats to subsequent implantation of the same tumors. They further demonstrated(8) that sarcoma tissue undergoing autolysis as a result of being sewn into a skin pocket, when transplanted into other rats of the same strain induced immunity against implantation of the tumor. We have observed that a number of Jax-C3H-(He) mice in which regression of lymphosarcoma 6-C3H-Ed was induced by intensive treatment with A-methopterin were immune(9). The present paper describes an extension of these experiments and others in which Jax-C3H mice of the Heston subline were immunized by means which prevented or interrupted the progressive growth of the tumor in these mice.

Materials and methods. Young adult (18-22 g) C3H (Heston subline) mice of both sexes and the Gardner lymphosarcoma 6-C3H-Ed, obtained from the Jackson Memorial Laboratory were used in all experiments. The tumor was carried for over 70 passages in these mice in our laboratory within the last 2 years, and in the passages and in numerous experiments in which these mice were implanted with it for control purposes, it has not failed to grow progressively and kill the recipients. The tumor was implanted by subcutaneous injection of tumor cell suspensions made by mincing 10-12-day-old tumors with scissors in saline. The suspensions were of such density that passage through a No. 22 gauge needle was accomplished. The mice were observed almost daily and were kept under the usual laboratory conditions, having a free access to food and water. A-methopterin was injected subcutaneously in 2 mg/kg/day doses of aqueous solution. Other methods are described in connection with the various experiments.

Experimental. In 6 experiments a total of 62 Jax-C3H (He) mice were implanted on the right flank with tumor cell suspensions. The dose implanted ranged in the various experiments from approximately 250,000 to 5,000,000 cells in 0.1 ml of saline. Beginning on the first to the fourth day following implantation, 2 mg/kg/day doses of A-methopterin were given. Dosage was continued for 7 successive days in recent experiments, and for 11

days in others(9). Two mice died during treatment, and of the 60 mice which survived, the tumor regressed in 21, usually between the 20th and the 35th day following implantation. Thirty-six untreated mice similarly implanted as controls died of progressive tumors between the 18th and 30th days depending upon the number of cells implanted. The 21 mice in which progressive tumor growth failed to occur were reimplanted with approximately 5 million tumor cells 6 or 7 weeks after the original implantation. Eleven of these mice which were not immune had never developed palpable tumor following the first implantation, whereas all of the 10 which were immune were mice in which regression of palpable tumors had taken place under the influence of A-methopterin. It is inferred from these observations that the tumor mass acted as the antigenic stimulus in inducing immunity.

Five Jax-C3H (He) mice were slowly injected into the tail with approximately 2.5 million tumor cells in 0.05 ml of saline suspension, care being taken to miss the tail veins. The injection was made equidistant between the base and the tip of the tail. All 5 of the mice failed to develop visible tumors. Four weeks after the implantation, the mice were challenged for immunity by subcutaneous injection of approximately 5 million tumor cells. Four of the mice failed to develop tumors, the fifth developed a pea size tumor which regressed. Six control mice injected with the same tumor cell suspension died of progressive tumors. The 5 immune mice were rechallenged after 2 weeks and again tumors failed to grow. The experiment was repeated using larger numbers of cells for implantation.

Twenty-five Jax-C3H (He) mice were injected into the tail with 5 million tumor cells in 0.1 ml of saline suspension. Eleven of these developed tumors in the tail which spread to the rump, killing the mice on or before the 42nd day after injection. Fourteen mice failed to develop tumors and were challenged on the 42nd day by subcutaneous injection of 5 million tumor cells into the flank. These 14 mice failed to develop tumors while each of 15 controls died of progressive tumors within 3 weeks after injection.

Eight Jax-C3H (He) mice were implanted in the same location in the tail as those described above. Approximately 10 million tumor cells were injected in 0.2 ml volumes of saline. Considerable force was used in injecting the dose and some oozing of fluid through the skin of the tail was observed. After 12 days, all of the mice had developed tumors which extended 0.25 inch in either direction from the point of injection. At 5 P.M. the blood supply of the tails was occluded by winding short lengths of No. 14 A.W.G. soft drawn copper wire around the tail about 0.5 cm from its base. The wire was made tight and secure by twisting the ends together with hemostats. Almost immediately the tail became engorged and the tumors discolored to a deep purple. The wires were left in place overnight. The next morning the tails were dry and blackened and the tumor severely damaged, being swollen, black and exuding thin cherry red liquid through fissures which had developed during the night. The tails and tumors retained this appearance even after the wires were removed at 9 A.M. The process was repeated at 5 P.M. on the same day with removal of the wires the following morning. At this time the tails and tumors were distinctly dry, and later in the day the lower portion of the tails were observed to have fallen off, breaking in the region where the tumors had been. No further treatment was given and the mice were observed for 10 days during which period one died. The others remained well although the tails became shorter, finally breaking at the point where the wires had been applied. Six weeks after the original implantation, these mice and 6 controls were injected subcutaneously in the flank with approximately 5 million tumor cells. Within 5 days pea size tumors developed in all of the mice. The tumors in the control mice grew progressively, causing death, while those in the test mice after remaining pea size for 4 days began to regress and all had disappeared by the 15th day. A second challenge implantation of tumor cells was given one month after the first; the tailless mice developed no tumors while each of 5 controls died with progressive tumors. The results of the experiments are combined and summarized in Table I.

TABLE I. Immunity to Lymphosarcoma 6-C3H-Ed Produced in C3H (He) Mice by Prior Implantation of the Tumor.

Experimental procedure	No. mice	Progressive tumors	Palpable tumors regressed	No. tumor developed	Total negative to first tumor implantation	Result of reimplantation of tumor	
						Not immune	Immune
A-methopterin, 2 mg/kg/day for 7 days	62 (2)*	39	11	10	21	11	10
Tumor inj. in tail (no treatment)	30	11		19	19		19
Tumor inj. in tail (tails tied off)	8 (1)†		7		7		7

* This tabulation includes mice in experiments previously described (9).
† Number in parenthesis died during treatment.

Summary. Experiments are described in which cells of lymphosarcoma 6-C3H-Ed implanted in Jax-C3H (He) mice, in doses which failed to induce tumors when implanted into the tail, in large doses which induced tumors that were destroyed by intermittent occlusion of the blood supply, and by regression of palpable tumors through intensive treatment with A-methopterin, led to development of immunity of the mice against subsequent progressive growth of the same tumor.

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Received July 17, 1952. P.S.E.B.M., 1952, v80.

The Mast Cell. Cortisone Action on Connective Tissue.* (19729)

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During recent years the effect of hormones on connective tissue has been the object of intensive research, particularly after it became known in 1949 that the adrenal cortex influenced a number of connective tissue diseases.

It is known(10,11) that cortisone inhibits new formation of connective tissue and that fibroblasts in healing wounds of treated individuals are smaller and more pyknic than in wounds of untreated subjects.

The effect on the connective tissue ground substance has attracted particular attention, because the formation of ground substance appears to be the essential condition for new formation of connective tissue. Since various observations(1,2,5) indicate that hyaluronic acid, which is an important component of the connective tissue ground substance, is formed by the mast cells, interest has also centered on the effect of hormones on these cells.

The present author(3,13) found that in

* This work was supported by grants from Eli Lilly & Co., Indianapolis, Ind., and The National Danish Association against Rheumatic Diseases.

TABLE I. Material and Dosage of Cortisone and ACTH.

	Cortisone,* total dose		ACTH,† total dose (internat.	
	No.	(mg)	No.	units)
Humans	32	750–2600	151	240–860
Rabbits	15	150–275	23	45–105
Mice	20	4,4–11	11	5–14
Guinea pigs	11	8–10	16	5–100

* Cortone (Merck).
† Acton (Frederiksberg Chemical Factories).

human patients the mast cells of dermal connective tissue decreased in number and became degranulated when exposed to the action of ACTH and cortisone. At the same time, the hyaluronic acid of the ground substance was reduced. Cavallero and Braccini(7) confirmed this finding in experiments on the rat.

Since an increased amount of hyaluronic acid is of great pathogenetic significance in various connective tissue diseases(4,9,12), such as rheumatoid arthritis, pemphigus, acute disseminated lupus erythematosus, keloids, fresh cases of scleroderma, and perhaps also in iridocyclitis and glaucoma, the author feels that the suppressive action on the formation of hyaluronic acid is an important link in the clinical effect of cortisone on these diseases. The transmitters of this action might be the mast cells of the mesenchymal tissues.

The mast cells in dermal connective tissue were thoroughly studied during administration of cortisone and ACTH to humans,† rabbits, mice, and guinea pigs.

The following was derived from these experiments: The number of mast cells decreases, and certain morphological changes take place. A varying number of cells are perfectly normal even during and after the treatment, but most of them exhibit characteristic changes, depending somewhat on the dosage. While the granules are normally of approximately uniform size and rather evenly distributed in the cytoplasm, administration of cortisone and ACTH is followed by more or less marked degranulation; the granules are distributed in masses of varying size. A number of the mast cells show a vacuolized cytoplasm; the partitions separating the vacuoles consist of a homogeneous or partly granular substance which stains partly metachromatically, partly orthochromatically with toluidine blue. In some instances it is difficult to make out the cell boundaries. The cells are irregular, ragged in shape, of varying size, but on the whole, small.

It seems evident that the reduction in the number of mast cells upon simple counting is due to the fact that many have lost their granules and thereby their histological characteristics.

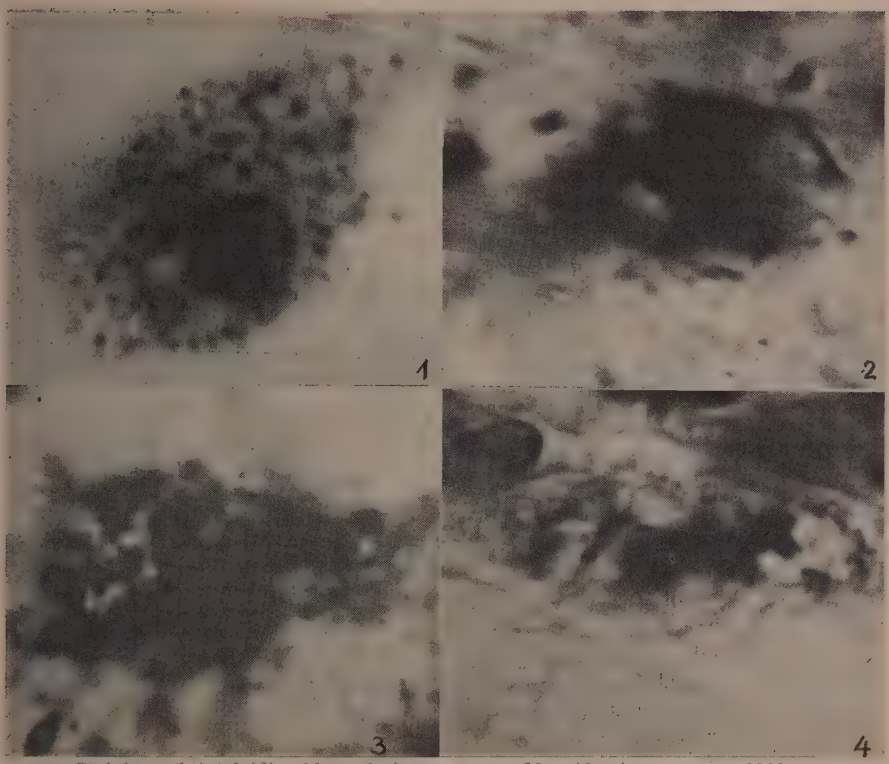
Discussion. While ACTH and cortisone alter the mast cells to shapes which appear degenerated, the thyrotrophic hormone of the pituitary has proved to increase the number of mast cells which become highly granulated at the same time that the content of acid mucopolysaccharides increases. The condition is reversible, yielding to thyroxin(6,8). Similarly, the pathological state observed during administration of cortisone is perhaps reversible. It has not yet been ascertained whether the altered mast cells perish or whether their activity may be revived by discontinuing the hormonal action.

Summary. During administration of cortisone to humans, rabbits, mice, and guinea pigs the number of mast cells in the connective tissue is decreased. At the same time alterations are observed in the number and distribution of their granules, in the shape and outlines of the cells, and vacuolization occurs.

Since mast cells are presumably the origin of hyaluronic acid in the connective-tissue ground substance, this suppressive action is important when viewed against the background of the clinical effect of cortisone on connective-tissue diseases in the increased amounts of hyaluronic acid play an essential pathogenetic role.

After these studies were completed, Frank Bloom (PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 651), has reported his experiments with malignant multiple mast cell tumors (mastocytomata). The tumors rapidly regressed and disappeared, and the neoplastic mast cells showed morphological changes like those described above.

† Unaffected skin taken from human patients suffering from various connective-tissue diseases.



Staining: 1% toluidine blue solution, aqueous. Magnification: approx. 3000 ×.

FIG. 1. Normal mast cell from human skin before administration of cortisone. The fine granules are uniformly distributed throughout the cytoplasm.

FIG. 2. After administration of cortisone, 1800 mg. Mast cell of dermal connective tissue showing vacuolization.

FIG. 3. After administration of cortisone, 1800 mg. Mast cell granules distributed in major and minor clusters and lumps, staining more or less intensely.

FIG. 4. After administration of cortisone, 1800 mg. Mast cell granules of irregular distribution, partly in the periphery, partly in lumps around the central nucleus. Vacuolization of the cytoplasm.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Strain and Sex Differences in Response of Inbred Mice to Adrenal Cortical Hormones.* (19730)

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Function of the adrenal cortex in both animals and man has been measured by the degree of eosinopenia which follows its stimulation(1,2). Speirs and Meyer(3,4), developed a bioassay for cortical hormones based upon this response in mice. Their technic is highly specific for corticoids, sensitive to doses as low as 0.5 μg and can be used for quantitative estimation. The assay is sensitive, rapid, and easily performed.

This report demonstrates that mouse strains differ in their responses to cortisone; thus for accurate assays it is imperative to use certain inbred strains, highly sensitive to cortisone, rather than mice selected at random.

Method. Male mice of 10 inbred strains or their hybrids, and females of 2 strains were tested by the method of Speirs and Meyer mentioned above. Inbred mice were adrenalectomized in a one-step operation and pellets of 11 desoxycorticosterone acetate implanted subcutaneously. A few days postoperatively, mice received a subcutaneous injection of epinephrine, followed in 4 hours by a dose of adrenal cortical hormone. Eosinophil counts were taken immediately before and 3 hours after injection of the corticoid. The percentage of decrease in number of eosinophils during this period was correlated with quantity of hormone injected.

Precautions were taken to stabilize environmental factors. Mice chosen were similar in age (2 months) and weight (20-25 g), and were bled usually the third and tenth day after adrenalectomy. For at least 20 hours previous to and during the run mice were kept in a dark, constant-temperature cabinet (80°F).

Preliminary experiments showed that the amount of adrenalin needed to eliminate non-specific responses varied with strains. Three to 5 μg for C57BR/cd, C57BR/a, C57BL/6, and BR/ed X BL/6 hybrids (called BBF_1) was found effective if the animals were newly adrenalectomized but not always so 3 to 4 weeks later. When the dose was increased to 20 μg all such non-specific responses were eliminated without producing highly toxic effects so this dose was adopted for these strains. With 129/Rr's 10-20 μg was used, DBA/1's required 30, and B ALB/c's 40 μg to eliminate non-specific responses. Larger doses such as 20 μg for 129's and 40 μg for B ALB/c's were probably responsible for occasional difficulty in bleeding or death.

Hormones, injected subcutaneously, were: .03 cc of cortisone acetate, dissolved in benzyl alcohol diluted with at least 9 volumes of sesame oil; .5 cc of cortisone (free alcohol), dissolved in distilled water.

Results. Strain differences. Strains injected with cortisone acetate and cortisone (free alcohol), are included in this comparison, because as Fig. 1 (plotted from data of Speirs and Meyer(4)) shows no significant difference occurred in response to these 2 types of hormone preparation in one strain tested—

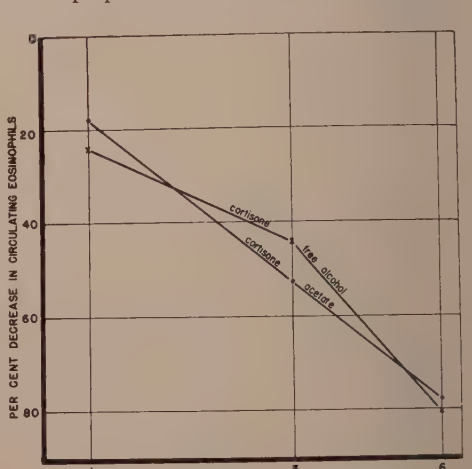

FIG. 1. Three-hour response of adrenalectomized male C57 brown ed mice to cortisone acetate; cortisone (free alcohol).

* Supported by a grant from the New York Foundation.

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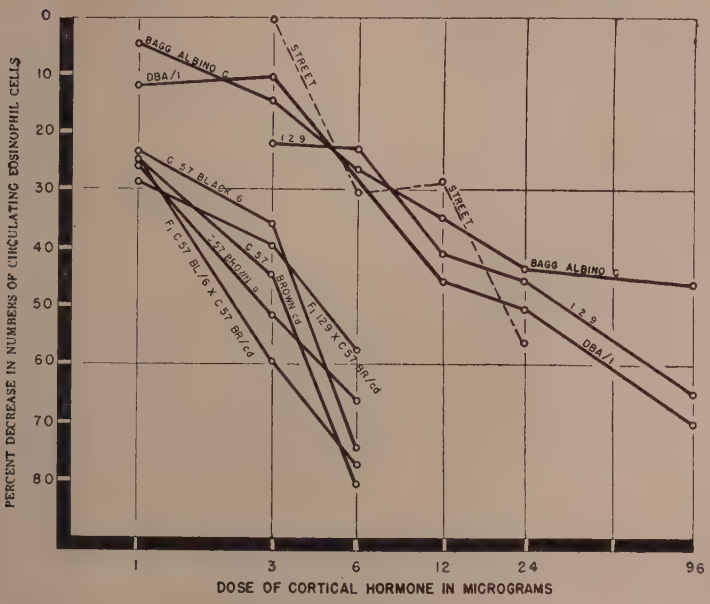

FIG. 2. Three-hour response of adrenalectomized inbred strains to adrenal cortical hormone.

C57BR/cd. The similar effects of these preparations have also been observed in BBF_1 (unpublished).

The average responses of 9 strains tested are plotted in Fig. 2. Table I gives the number of animals per point, standard deviation, standard error, and slope of each curve. (Method given by Dorfman(5)). From the figure it is apparent that responses fall into 2 distinct groups. 1. Sensitive strains: respond significantly to 1 μg and maximally to approximately 6 μg of cortisone. (C57BL/6, C57BR/cd, C57Br/a, BBF_1, F_1 129 X BR/cd). 2. Insensitive strains: require 3-6 μg for a significant response and at least 96 μg to approach maximum eosinopenia. (129/Rr, DBA/1, B ALB/c, Street).

An additional type is represented by the strain Small, in which cells are too few to use in calculations. Of 24 counts made, 64% had less than 60 eosinophils per mm^3 blood.

Variability of response according to dose and strain is brought out in Table I. The standard deviation of most groups of animals in sensitive strains is comparatively small (from 3.5 to 16) for the high dose of 6 μg. For the same dose, in insensitive strains, it is from 16-23. Strains such as the Street, F_1 BR/cd X 129, and C57BL/6 seem more variable than others.

Sex differences. Table II shows the per cent decrease in circulating eosinophils of females (BBF_1, C57BR/cd) to cortisone (free alcohol) and cortisone acetate is less consistent than that of males. In addition, their standard deviation is generally greater except for the 3 μg injection of cortisone acetate, but this response is much smaller than for male animals.

Eosinopenia and behavior under stress. The C57BR/cd is an active mouse which jumps, squeals, and tries to bite when handled. It gives maximum eosinopenia after 6 μg of cortisone—a highly sensitive response. On the other hand, the 129/Rr although active, is a mild-mannered mouse which is easily handled and rarely if ever attempts to bite. Thus it is a good laboratory animal. Furthermore its light color makes tail veins easy to see and it bleeds well. Unfortunately for assay work, it is less sensitive to cortisone, requiring a dose 16 times as large as the Brown to give maximum eosinopenia.

Fig. 3 shows the hourly response of normal

TABLE I. Standard Deviation, Standard Error, and Slope of Response of Some Inbred Mouse Strains Injected with Cortical Hormone.

Strain	Cortisone used	Dose 1 μg Avg resp.	1 μg S.D.	1 μg S.E.	3 μg Avg resp.	3 μg S.D.	3 μg S.E.	6 μg Avg resp.	6 μg S.D.	6 μg S.E.	12 μg Avg resp.	12 μg S.D.	12 μg S.E.	24 μg Avg resp.	24 μg S.D.	24 μg S.E.	96 μg Avg resp.	96 μg S.D.	96 μg S.E.	Slope
C57 BR/a	Acetate	25	18.5	7.7	51	10.9	5	66	16.5	7.5										41.5
C57 BR/cd	Free alcohol	24	22.8	9.5	44	27	11	80	3.4	1.4										68.4
C57 BL/6	Acetate	23	35.9	15	35	31.6	12.8	74	15.4	5.9										76
BBF_1	”	22	14.4	6.5	59	23	8.8	77	8.8	3.4										81.8
129 × BR/cd	Free alcohol	28*	25.7	14.8	39	18	6.4	57	21.4	8.9										86.9
B ALB/c	Acetate	4	8.5	4	14	17.5	7.3	26†	16.2	5.1	34	11.8	5	43	13.4	5.6	46†	27.7	8.7	12.7
DBA/1	”	11†	13.7	4.6	10	10	3.6				45†	18.5	8.4	50	6	2.7	70	5.9	2.7	20.3
129/Rr	”				21	16.1	6.7	22	22.9	9.5	40	20	6.2	45†	15.1	5	66	19.3	6.9	23.2
Street	”				0	0	0	30	23.1	9.6	28	31.6	13	56	13.9	6.3				64.9

Note: Usually 6 animals per point.
* 3 animals.
† 9–12 animals.

TABLE II. Sex Differences in Response to Cortisone.

Strain (adrenalectomized)	Dose in μg cortisone	Avg % decrease in eosinophils ♂	Avg % decrease in eosinophils ♀	No. of animals ♂	No. of animals ♀	Stand. dev. ♂	Stand. dev. ♀	Stand. error ♂	Stand. error ♀
F_1 hybrid	1 (free alc.)	19	20	7	6	19.1	17.4	7.3	7.3
	3 ” ”	62	40	7	6	11.6	16	4.8	6.7
	6 ” ”	73	59	6	6	7.9	15.1	3.4	6.3
C57 BL/6 × C57 BR/cd	1 (acetate)	14	7	8	9	15.6	12.4	5.6	4.3
	3 ”	48	12	6	6	21.4	7.1	7.6	3
	6 ”	64	37	12	11	15.1	24.2	4.3	7.3
(BBF_1 C57 BR/cd	1 (free alc.)	7	28	6	4	11.8	24.4	5	12.2
	3 ” ”	64	78	3	3	14.8	12	8.7	7
	6 ” ”	80	87	6	4	3.5	4.8	1.4	2.4

animals to stress. Under mild stress of handling (warming and taking blood from a nick in the tail vein) the number of circulating eosinophils in the 129 is not disturbed nearly so much as in the Brown. The 129 shows a decrease for only one hour, rather than 4, and reaches a maximum of —31% in contrast with —85% in Browns. When stress in 129's is increased by injecting 10 μg of epinephrine, their response is almost identical with that of the mildly stimulated Browns. If further increased to 20 μg, the same rapid decrease occurs in 4 hours, but the numbers remain low for a longer period, with a decrease of 64% still present at 8 hours (not plotted).

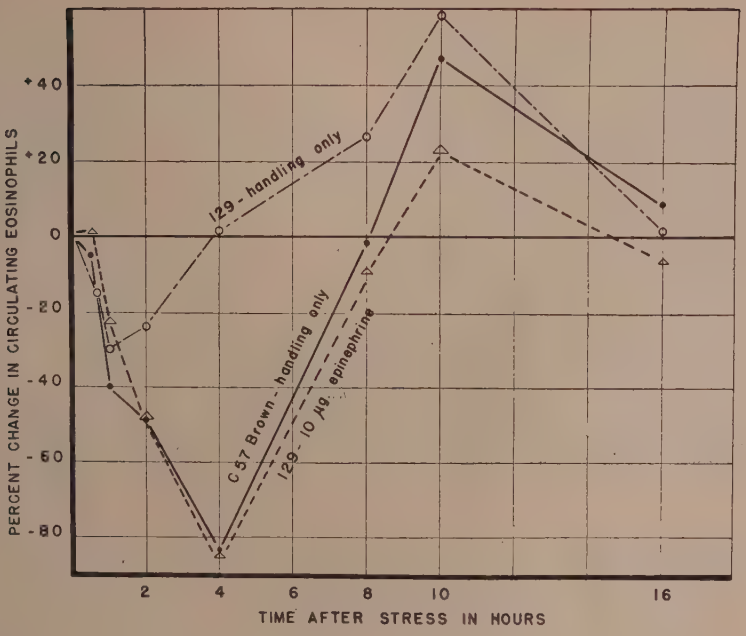

FIG. 3. Hourly response of normal 129 and C57 brown cd male mice to stress.

It should not be concluded that quiet disposition and poor sensitivity to injected cortisone are related. The DBA, an extremely active strain, is one of the least sensitive to injected cortisone.

Discussion. Two factors to be considered in comparing strains are sensitivity and variability.

When a strain sufficiently sensitive to detect physiological amounts of hormone is used for assay, the work and sources of error inherent in concentration processes may be reduced or eliminated. Sensitive strains frequently give satisfactory responses when non-extracted materials such as .5 cc urine from ACTH-treated patients is injected.

Less variability occurs as maximum eosinopenia is approached. Thus in sensitive strains 4 to 6 animals injected with 6 μg of cortisone give a very strong and reliable point, whereas smaller doses generally give values with more variability. With insensitive strains 24-96 μg are required to produce an eosinopenia large enough to manifest the reduced variability. (Note the exception that B ALB/c's are most variable at 96 μg).

Female mice have been found to respond like males by some workers. A few normal females (Tumblebrook Farm strain) gave an eosinopenia similar to that of males when injected with ACTH or foreign protein(6), and adrenalectomized females have been used for assay(7). Experience at the Jackson Laboratory indicates that females sometimes give results similar to males, but generally they are less reliable (Table II).

In choosing mice for the cortisone assay based on a decrease in circulating eosinophils, it must be obvious that non-inbred strains are less satisfactory because responses differ according to genetic constitution. In inbred strains, where individuals are genetically identical, this serious variable is eliminated. Their use also markedly reduces the number of controls necessary for assays.

After considering all factors, the BBF_1 male seems the best animal to use for assay work. Although it is rather wild and its veins are difficult to see due to the dark tail, it is very sensitive to cortisone, and its hybrid vigor shown in large litter production and resistance to toxic materials makes it economically the

preferable strain.

Summary. Adrenalectomized mice varied in response to cortisone according to genetic constitution. Some strains were: a) highly sensitive. They responded significantly to 1 μg of cortisone, and maximally to 6 μg. b) Relatively insensitive. They responded to 3-6 μg but required 16 times the dose of a sensitive strain to approach maximum response. c) Too low in eosinophils to be practicable in calculations. Males were generally more reliable than females. Normal animals of 2 strains differed in response to mild stress. F_1 hybrid males from C57BR/cd X C57BL/6 are recommended for the assay described.

We are indebted to Ciba Pharmaceutical Co. for Desoxycorticosterone acetate pellets used in this study, and to Mr. J. Sullivan for technical assistance.

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Received July 18, 1952. P.S.E.B.M., 1952, v80.

Effects of Antibiotics on Daphnia.*† (19731)

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In his recent studies of hemoglobin formation in Daphnia, Fox has devised a method for estimation of the blood pigment in the living animal and shown that its concentration increases in response to lowering of the oxygen tension of the environment(1). It would appear that the hemoglobin system of such small aquatic animals may provide a useful tool for the study of certain problems of hemoglobin metabolism, among them aspects of nutrition. Interest in the adaptation of Daphnia to this kind of investigation has led to an attempt to cultivate the animals free of the natural microflora of the intestine, which, through synthetic or degradational enzymatic activities, could preclude certain types of experiments. At the same time, it was recognized that success in the attempt might well create a nutritional problem, inasmuch as living bacteria in the gut will doubtless be necessary for normal growth of numerous species, including Cladocera(2), until their vitamin and other requirements have been more fully defined. Accordingly, it was decided to try to eliminate bacteria and fungi by the use of various combinations of antibiotics. The results that relate especially to the nutrition of Daphnia will be reported elsewhere. Meanwhile, in the course of the work it appeared that the tolerance of Daphnia for some chemotherapeutic agents parallels that of man. This fact, together with the several convenient qualities of Daphnia as a laboratory subject suggested that the findings might be of interest from a pharmacological point of view, and could perhaps be of use to those concerned with the toxicity of antibiotics.

An obvious deficiency of the microcrustacea

* This investigation was supported by a Research Grant from the National Institutes of Health, U. S. Public Health Service, and by the Holmes Foundation, Inc.

† The authors are indebted to Prof. H. Munro Fox for advice on cultivation of Daphnia, and to Prof. S. R. Detwiler for initial supplies of Daphnia. Thanks are likewise expressed to Dr. Gladys Hobby of Chas. Pfizer & Co. for supplies of some of the agents and for useful suggestions.

in experimentation is their size, which makes parenteral injection of materials with ordinary equipment virtually impossible. There are, on the other hand, the familiar advantages that Daphnia are readily cultivable in large numbers within a small space and that they exhibit a number of easily appreciable indices of injury, while their transparency permits direct microscopic examination in life of circulating blood cells and viscera. Moreover, metabolic rates, in terms of oxygen consumption, may be readily determined over a period of hours, or longer, by the use of Fox and Wingfield's modification(3) of the Winkler method. These points are illustrated in the data, which compare the toxicities of a number of antibiotics for Daphnia.

Methods. The strain of *Daphnia pulex* employed was cultivated from a single female. The maximum length reached is about 2.5 mm. Routine cultures of Daphnia were maintained in tanks at room temperature, about 23°C while all experiments were conducted in 250 ml bottles, each containing about 150 ml of water, which were placed in a constant temperature incubator at 20°C. Considerable difficulty was encountered in finding a water of suitable composition for certain experiments, notably those in which Daphnia were kept without food. It appears that a few workers have found it possible to keep Daphnia alive in pure distilled water for as long as 10 days (4). Such tolerance is by no means the rule for fresh water crustacea; wide variations in ability to retain chloride(4) and in requirement for cations(5) occur and may involve strain differences. The strain of Daphnia used here survived for less than 24 hours in water triple-distilled with permanganate. Nor did the animals live longer in tap-water bubbled vigorously with air and having a total content of solids of about 75 p.p.m. and a pH of 7.0. After these findings, trials were made of natural hard waters containing some 200 p.p.m. of total solids, of which there were about 20 p.p.m. each of calcium and magnesium, and having a pH of about 7.8 after equilibration with air. In such waters Daphnia usually survived regularly for 8 to 10 days without food. It was also found that an artificial water made up to contain calcium superphosphate permitted regular survival of most of the animals for 8 to 10 days when starved and it was decided to use this water routinely. The composition is as follows: To each liter of tap water are added 1) 60 mg $CaSO_4$, 2) 80 mg calcium superphosphate, 3) 80 mg magnesia alba. The mixture is bubbled vigorously with air overnight and filtered. Final pH is about 7.9; the electrolyte conductivity corresponds to about 120 p.p.m. NaCl.

Chemotherapeutic agents were added in varying concentrations to the water and each test was run in duplicate in 250 ml bottles which were then seeded with about 12 Daphnia. For each experiment controls were set up in water alone; these animals invariably survived for more than a week even though not fed. The end-point generally employed to estimate toxicity was death of all of the Daphnia. The experiments were terminated on the seventh day since the controls usually died shortly thereafter. Measurements of pH with the glass electrode showed variations only within the range 7.8 $\pm$ 0.1, *i.e.,* differences unlikely to influence the results. Where feeding was undertaken a suspension was used of *Chlorella vulgaris* grown on agar slants. The suspension was adjusted to a standard optical density in a Coleman spectrophotometer and a fixed amount added to each bottle at intervals of a few days. Oxygen content of the water was measured by the modification of Winkler's method described by Fox and Wingfield(3). The sampling syringes employed differed only in detail from theirs. The use of phosphoric acid as recommended by them was found to give somewhat lower readings than predicted values when tests were performed on distilled water equilibrated with air. The substitution of 20% HCl, employed by Krogh(6), overcame this difficulty, and it was then found possible to obtain values for oxygen content having an error of not more than 2% even when the sample was about one ml. All of the determinations were performed in duplicate. The solutes employed in these experiments did not introduce any significant error.

Results. In Table I is set forth the comparative toxicity of a number of agents in

TABLE I. Survival in Days of Starved Daphnia in Varying Concentrations of Chemotherapeutic Agents.

Group	Antibiotic	Conc. in μg/ml of water 50	25	12	6
I	Penicillin G Terramycin (HCl) Chloramphenicol Sulfonamides*	7	7	7	7
II	Streptomycin sulfate	3	4	7	7
	Dihydrostreptomycin Cl.	2	5	7	7
	Polymixin B	2	3	3	5
	Neomycin sulfate	3	3	5	6
III	Actidione	—	—	1	1
	Bacitracin†	7	7	7	7
IV	Thiolutin‡	1	1	7	7
	Rimocidin‡	1	2	5	6

* Includes sulfadiazine, sulfamerazine, sodium sulfathiazole, succinyl sulfathiazole.

† Containing 58.2 units/mg.

‡ Kindly supplied by Dr. Gladys Hobby of Chas. Pfizer & Co.

terms of length of survival of the exposed Daphnia, which were starved except for the unknown contribution to their nutrition that may have been made by the antibiotics themselves or derived from them by bacterial action. The concentrations include those that may be reached in the tissues when the substances are employed in man. The drugs are arranged arbitrarily in groups. In Group I are those compounds in common clinical use which are absorbed at least moderately well from the human intestinal tract and which exhibit comparatively little immediate toxicity for man. Aureomycin could not be included in the studies because it proved to be too unstable. Group II includes agents which are known to be rather less well tolerated by man. The substances in Groups III and IV are separated from the others merely for convenience of appraisal in what follows. It may be seen that penicillin, terramycin, chloramphenicol, and the sulfonamides exhibited no evident toxicity for Daphnia, which showed normal activity throughout the duration of the experiments. No abnormalities were noted in their appearance on microscopic examination. All of these agents are relatively stable at 20°C for several days, with the exception of penicillin. In separate experiments penicillin was added to the water every 2 days, each time in amounts sufficient in themselves to restore fully the original concentration, yet no evidence of toxicity was found. Nonetheless, it is of course possible that the apparent innocuity represents failure of absorption.

The streptomycins (Group II) were lethal in higher concentrations, but at what may be considered therapeutic levels, *i.e.*, 6 to 12 μg/ml, they did not affect survival or behavior. Polymixin and neomycin in man may cause renal damage(7,8) and neomycin may injure the 8th nerve as well(8). The same agents appeared to be highly toxic for Daphnia, which succumbed to their action in less than a week.

Of particular interest is the finding that neomycin exerted a specific pharmacological action on the gut. After about 24 hours' exposure to concentrations of 6 to 12 μg/ml, microscopic examination disclosed a very tight constriction of the intestine, always at about the junction of the first and middle thirds. No similar effect was noted with any of the other drugs tested.

Thus far there is substantial agreement for each antibiotic considered between toxicity for man and for Daphnia. In Group III is bacitracin, apparently harmless to Daphnia, yet known to be nephrotoxic in the human subject(9). But bacitracin is quite without effect clinically if given by mouth because it is destroyed in the gut, and this may well hold also for Daphnia. Actidione was by far the most poisonous material tested. In experiments not included in the data of Table I it could be shown that concentrations as low as 1 μg/ml killed Daphnia in a few days. The substance has been employed in man to a limited extent, and the acute toxicity is described as nausea (10). For rodents actidione is highly poisonous, whether instilled into the stomach or given intravenously(11); the lethal dose appears to lie between 1 and 2.5 mg/kg.

Group IV includes only 2 fungicidal agents for which data as to effects on other species are not available, although they are certainly not harmless(12).

For the more toxic antibiotics, in addition to the experiments with starved Daphnia a corresponding series was run in which the animals were fed chlorella. No significant length-

TABLE II. Oxygen Consumption of Daphnia.

Bottle No.	1*	2	3	4	5†	6
No. of Daphnia	0	42	50	57	0	0
Streptomycin, μg/ml	10	5	10	0	0	0
Chlorella	+	+	+	+	+	0
			Oxygen content of water, cc/l			
March 12, p.m.	6.05	6.04	6.06	5.99	6.08	6.03
March 14, p.m.	5.00	4.06	3.85	3.14	4.32	5.39
Oxygen loss	1.05	1.98	2.21	2.85	1.76	.64
Calculated		−1.05	−1.05	−1.76		
Total oxygen loss from Daphnia		.93	1.16	1.09		
			Oxygen consumption, μl/hr			
Per Daphnia		.11	.12	.10		
Per μg non-exoskeletal N			.05			

* Bottle 1 represents respiration of chlorella and bacteria; the value for this oxygen loss is subtracted in bottles 2 and 3.

† Bottle 5 represents respiration of chlorella and bacteria; the value for this oxygen loss is subtracted in bottle 4.

ening of life span was seen as a result of feeding.

Having examined the effect of antibiotics on the survival of Daphnia, it seemed worthwhile to find out whether the exhibition of an antibiotic might produce a measurable influence on metabolism. For this purpose it was judged best to employ an agent which in high concentration was demonstrably harmful, and therefore absorbable, but in lower concentration was seemingly innocuous. Streptomycin has been shown to have these qualities. Moreover, it was found that a concentration of streptomycin sulfate of 10 μg/ml did not, over a period of 2 weeks, affect nutrition or egg-laying in Daphnia that were fed living *Chlorella vulgaris*.

In Table II are recorded the data for oxygen consumption of Daphnia in the presence of streptomycin and in control preparations over a period of 2 days. The mean wet weight of Daphnia after drying briefly on filter paper was 0.3 mg. Eleven molted exoskeletons were found by micro-Kjeldahl analysis to contain 6 μg N. Three separate groups of 25, 28, and 28 whole Daphnia contained, respectively, 55, 73, and 78 μg N, or a mean of 2.5 μg per animal. The mean non-exoskeletal N is thus estimated to be 2 μg, and the rate of oxygen consumption may therefore be expressed in terms which permit comparison with existing data for related species(13). The oxygen loss given in bottle 5 represents the respiration of bacteria and chlorella (the preparations were kept in the dark). This value is regarded as a control and is subtracted from the total oxygen loss given in bottle 4 in order to gain an approximation to the amount of oxygen used that is attributable to respiration of Daphnia. The preparations represented in bottles 1, 2, and 3 all contained streptomycin. Otherwise, bottle 1 corresponds to control bottle 5, and likewise serves for the measurement of oxygen consumption of bacteria and chlorella. The oxygen loss is less than that in bottle 5, presumably because streptomycin has produced some bacteriostasis. Despite the obvious limitations and assumptions involved there is good agreement in the figures for oxygen consumption of Daphnia with and without added streptomycin, and therefore no evidence that streptomycin in any way has augmented or depressed respiration. In terms of non-exoskeletal N, the oxygen uptake of Daphnia under these experimental conditions appears to be in the neighborhood of 5×10^{-2} μl per μg N per hour.

Discussion. It is of interest to compare this value with those obtained by Zeuthen for marine microcrustacea of similar size, in acute experiments employing either the Cartesian diver respirometer or the Winkler method

(13). Amongst animals examined by Zeuthen were copepods about 0.85 mm long, each containing some 0.5 μg N. They respired at a rate of 7 to 8 x 10^{-2} μl O_2 per μg N per hour. Gammarus, containing about 1 mg N, consumed approximately 2 to 4 x 10^{-2} μl O_2 per μg N per hour. Daphnia is intermediate in size and the value calculated above for its oxygen consumption corresponds well to what might be anticipated from Zeuthen's data.

It would be interesting to know whether the tolerance of Daphnia for the agents listed in Group I, Table I, represents truly lack of toxicity or, rather, impaired absorption. It is probably a fair assumption for the relatively stable substances of known structure, *e.g.*, chloramphenicol and the sulfonamides, that some absorption from the gut took place. The same cannot be said for penicillin and terramycin. It might be possible to examine the question directly by placing Daphnia, carefully washed, on agar plates seeded with bacteria of known sensitivity, and so devise a modification of the inhibition-zone technic for measuring antibiotic concentrations. Such an undertaking lies beyond the scope of the present work.

The findings overall indicate a parallelism between toxicity of certain antibiotics for Daphnia, in terms of survival, and toxicity for man, in terms of untoward reactions that have limited their use. This is perhaps not surprising and in itself suggests no more than that Daphnia might profitably be employed as a test animal, bearing in mind the advantages and disadvantages already noted. The comparative fitness of Daphnia for such practical purposes could take on added significance if it were shown for any antibiotic that its potential harmful effects were reflected experimentally more clearly in Daphnia than in the rodents commonly used. The point may be illustrated by an examination of neomycin. The initial paper describing the substance reported that it exhibited little or no toxicity for animals(14). Yet within a year clinical trials indicated that of 32 patients receiving the drug in whom serial urinalyses were done, no less than 24 (75%) showed casts. Moreover, eighth nerve injury was encountered, and the conclusion was drawn that the material should probably not be released for general use(8). No doubt more exacting animal experiments would have narrowed this discrepancy. But it is noteworthy that in Daphnia neomycin demonstrated at once and unmistakably, a high degree of toxicity as judged both by its shortening of life and by its apparently specific action on the gut, which was easily seen *in vivo*. These considerations provide evidence that in particular instances a more successful prediction of potential toxicity can be made from simple experiments in Daphnia than from studies in higher animals.

Summary. 1. The survival of Daphnia following exposure to varying concentrations of chemotherapeutic agents was studied. 2. The toxicity of the antibiotics for Daphnia in terms of survival showed a correspondence to their acute toxicity for man, in terms of untoward reactions. 3. An apparently specific pharmacological action of neomycin on the gut of Daphnia is described. 4. The oxygen consumption of Daphnia was found to be about 5 x 10^{-2} μl per μg N per hour. The rate was not altered following addition of 10 μg/ml of streptomycin. 5. The use of Daphnia as a test animal for screening antibiotics is considered.

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Received June 19, 1952. P.S.E.B.M., 1952, v80.

A Growth Factor for *L. citrovorum* Synthesized by Hemopoietic Tissue Reversing Aminopterin and Chloromycetin Inhibition.* (19732)

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When suspensions of leukocytes or myeloid elements of bone marrow obtained from human sources are incubated with folic acid under conditions similar to those described for rat liver tissue(1,2), a substance is formed which stimulates the growth of *L. citrovorum* without the addition of folinic acid† but which cannot be assayed accurately(3) using this vitamin as a standard curve. When turbidity readings are plotted against increasing concentrations of leukocyte extracts, a growth response curve is obtained which is distinctly different from folinic acid (Fig. 1). Treatment with hog kidney conjugase(4) does not alter the type of response curve, an indication that the factor is not a glutamyl conjugate of folinic acid(5).

Since this factor is produced by an enzymatic process in hemopoietic tissue in the presence of folic acid, it has been tentatively designated as *hemofolin* for reference purposes. Although it would appear that hemofolin is a hitherto undescribed derivative of folic acid, the possibility remains that it is a product of some reaction in which folic acid is an obligatory participant.

Hemofolin has also been characterized as to its role in reversing the inhibition of *L. citrovorum* produced by 4-aminopteroylglutamic acid (Aminopterin) and by chloramphenicol (Chloromycetin). The results in Table I show that both hemofolin and folinic acid reverse Aminopterin inhibition. In the case of some levels of chloromycetin inhibition, however, whereas folinic acid is inactive as a reversing agent, hemofolin will promote growth of the bacteria in the presence of the antibiotic.

These data assume importance from several standpoints. In the first place, the differing behavior of hemofolin and folinic acid in chloromycetin inhibition - reversal studies serves to emphasize the distinctiveness of hemofolin and to offer a further means of distinguishing it from folinic acid. The fact that hemofolin can overcome the growth inhibition of chloromycetin constitutes evidence relating the action of this antibiotic to folic acid metabolism in bacteria. Some preliminary results have indicated that such a relation exists in animal tissue as well(6).

These inhibition studies also suggest that the metabolically active form of the folic acid vitamins in *L. citrovorum* may be more closely identified with hemofolin than with folinic acid. The reversal of aminopterin by both folinic acid and hemofolin and of chloromycetin by hemofolin alone is interpreted to mean that folinic acid is a metabolic antecedent of hemofolin.

Attempts at concentration of hemofolin have been hampered by the extremely small amounts of the substance available. It has not been demonstrated to occur in leukocytes prior to incubation with folic acid, in liver tissue before and after folic acid incubation, or in kidney or intestine although its presence in some of these substances may be masked by the preponderance of other vitamin forms, There is some evidence that it occurs in certain types of yeast extract. It is hoped that the development of specific inhibition analysis technics will enable a more critical evaluation to be made as to whether hemofolin normally exists in natural materials.

For the most part, sources of the hemofolin-producing enzyme have been the peripheral leukocytes of persons with chronic leukemia. Assigning a value of 10 units to the amount of hemofolin necessary for optimal growth in *L. citrovorum*, from 250 to 700 units are formed per ml of leukocytes under the assay conditions employed. These include addition of folic acid and ascorbic acid with incubation

* Supported in part by a grant from Parke, Davis and Co.

† We are indebted to Dr. William Shive of the University of Texas for this material.

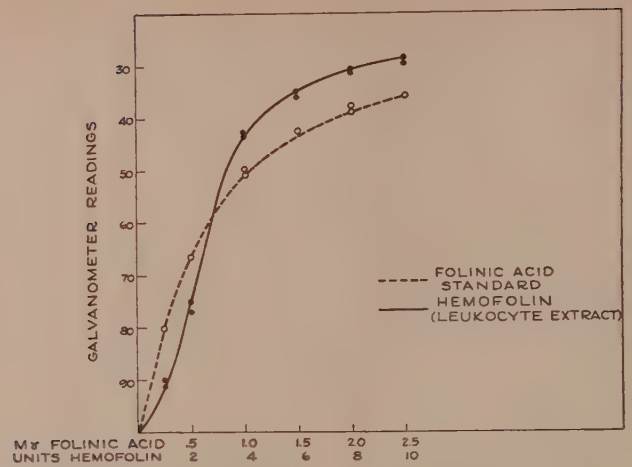

FIG. 1. 24 hr growth response of *Leuconostoc citrovorum* to a substance contained in leukocytes incubated with folic acid (hemofolin).

at 38°C in phosphate buffer pH 7.0. The enzyme for producing hemofolin has also been found in splenic tissue from various animals.

From the evidence presented as to its mode of formation, it might be presumed that hemofolin is closely related to or identical with an active coenzyme form of folic acid which is of special significance in hemopoietic cells. In this connection, it might be suggested that the ability to produce hemofolin is lacking in pernicious anemia. However, experiments have shown that bone marrow cells from untreated pernicious anemia patients can synthesize hemofolin where folic acid is the substrate.

TABLE I. Reversal of Aminopterin and Chloromycetin Inhibition of *L. citrovorum* by Hemofolin.

Inhibitor	Concentration, γ/tube	Amt for ½ max growth: Folinic acid, mγ/tube	Amt for ½ max growth: Hemofolin concentrate, units/tube
None	—	1.25	5
Aminopterin	25	3.40	10.9
	50	5.80	25
Chloromycetin	40	>250	14.4
	90	>250	51.8

Summary. An apparently new growth factor for *L. citrovorum* has been described. It is differentiated from folinic acid on the basis of growth-response curves, and reversal of chloromycetin inhibition. This factor is produced by homopoietic tissue incubated with folic acid and has been called hemofolin.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Study of Antifoaming Agents and Preliminary Evaluation of their Use in Experimental Pulmonary Edema. (19733)

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In some patients who die in an attack of pulmonary edema it seems likely that an important cause of death is asphyxia due to mechanical interference with gaseous interchange due to foam which fills the airways. If this foam could be collapsed, the volume of the resulting liquid would be only a small fraction of the original foam volume. The airways would be cleared to the extent of the difference between these 2 volumes. In certain cases of pulmonary edema the resultant increased effectiveness of administered oxygen could be of great value.

The first phase of this investigation was concerned with the testing of various antifoaming substances on transudate fluid *in vitro*, the second phase with an evaluation of their use in experimental pulmonary edema.

I. *Comparison of antifoaming activity of various substances in vitro. Method.* Foam was produced from ascitic fluid which is considered similar to pulmonary edema fluid. The fluid was poured into a vertical glass cylinder, closed below by a rubber stopper, perforated to permit the passage of a fritted glass tube. Oxygen was bubbled through this tube into the fluid at a constant rate until a given volume of foam was made. Oxygen was then turned off and the rate of foam collapse measured. The foam was then remade to the original volume, the antifoaming agent to be tested was dripped into the tube from a small syringe through a 20-gauge needle and the rate of collapse again measured. The rates of foam collapse before and after addition of the antifoaming agent were expressed as a ratio and termed "Foam Destruction Index" (I_1). Finally, oxygen was once more bubbled through the fluid which now contained antifoaming agent and the rates of foam formation before and after addition of the agent were compared; their ratio was termed "Foam Prevention Index" (I_2). The indices I_1 and I_2 measure the effectiveness of the antifoaming agent in dissolving foam and preventing foam formation, respectively. This method, modified from Bickerman(1), was found to yield reproducible results if care was taken to carry out every step in a suitably standardized manner.

TABLE I.
Antifoaming Activity of Various Agents.

Antifoaming agent	Foam destruction index* I_1	Foam prevention index* I_2
Control	1	1
1. Ether	2	1
2. Ethyl alcohol	2	—
3. Octylic "	16	—
4. DCAA 5% in ether	4.5	1.1
5. " 10 " "	4.5	1
6. TBP 1% in water	2	—
7. " 100%	38.5	—
8. Aerosol 18 5% in water	1	—
9. DCAA 5% + Aerosol 18 2.5% in ether	11.5	2.2
10. DCAA 6.6% + Aerosol OT 3.3% in ether	25	1.8
11. DCAA 3.3% + Aerosol 18 1.7% + TBP .34% in ether		
12. DCAA 6.6% + Aerosol OT 3.3% + TBP .67% in ether	37.5	8

* Each figure is the mean of several determinations. For abbreviations, see text.

Results. Some representative findings obtained by the method described are summarized in Table I. The relative ineffectiveness of ether and ethyl alcohol, pharmacologically suitable, in comparison with octylic alcohol, entirely unsuited for inhalation, prompted a search for agents combining clinical usefulness with efficacy. DC Antifoam A (DCAA), a methylpolysiloxane, has been shown to be non-toxic(2). However, solutions of DCAA in ether are only moderately effective. Tributylphosphate (TBP), although a powerful antifoamer, was irritating on inhala-

TABLE II. Effect of an Antifoaming Agent (DC Antifoam A) on the Development of Adrenaline-Induced Pulmonary Edema in Rabbits.

Agent	Wt, kg	Total adrenaline, mg	Degree of pulm. edema	Lung:body ratio*	Survival time,† min	Mode of death	Pulm. edema Present	Pulm. edema Absent
				Control animals				
Norm. saline	2.5	1.8	2+	.60	20	S	+	
	2.7	2	3+	.84	20	S	+	
	2.3	2	2+	.89	35	S	+	
Ether	2.1	2.5	1+	.64	20	N	+	
	2.4	2.5	2+	.85	10	S	+	
	2.3	2.5	2+	.64	15	S	+	
	2.5	2.5	2+	.73	15	N	+	
None	2.5	2	3+	.80	20	S	+	
	2.5	2.5	3+	1.10	15	N	+	
				Treated animals				
5% DCAA	2.9	2.5	0	.42	15	S		+
	2.4	2.5	?	.56	15	i.v. air		+
	2.5	1.5	2+	.74	15	S	+	
	2.4	2.5	2+	.87	15	N	+	
	2	2	2+	.95	10	S	+	
10% DCAA	3.4	3	?	.44	25	E		+
	2	2.5	0	.52	15	N		+
	3.3	2.5	0	.34	15	S		+
	2.6	2.5	2+	.85	15	N	+	
	2.2	2.5	3+	1.13	15	N	+	

* Lung:body ratio—lung wt × 100/body wt. † Measured from start of adrenaline.
T—trace. S—spontaneous. N—nembutal. E—electrocuted.

tion unless diluted to at least 1% at which point it lost most of its potency. Combinations of DCAA and certain detergents (Aerosol 18 and Aerosol OT), with or without TBP, enhance antifoaming activity to a remarkable extent. Although Aerosol OT (Dioctyl sodium sulphosuccinate) appears to be non-toxic on ingestion(3), inhalation of related aerosols in concentrations over 0.1% was found to be injurious to the lungs(4). The usefulness of this method in determining the antifoaming activity of various agents is suggested by these data.

II. *Effect of antifoaming agent (DC antifoam A) on adrenaline-induced pulmonary edema in rabbits.* Five and 10% solutions of DCAA in ether were the agents studied. More potent agents (Table I) were not used because of their tendency to produce lung damage. Pulmonary edema was produced by the slow intravenous injection of dilute adrenaline solution (0.25 mg per ml). This modification was found to reduce death rate within the first 10 minutes following injection(9,10). Adrenaline and antifoaming agent or control solution were administered in a standard manner. Male rabbits were placed in a holding box and antifoam or control solution directly sprayed into the mouth through a Devilbiss atomizer. After the animal received 3 to 5 squirts per minute for 5 minutes the adrenaline solution was injected into an ear vein at the rate of one ml per minute. The injection was continued until the animal either had received 10 ml (2.5 mg) of adrenaline solution or developed manifest pulmonary edema. During this period the spray was continued at 2 minute intervals. The solutions used were stained with methylene blue and examination of the lungs postmortem showed that satisfactory penetration of the sprayed solution had been obtained. The rabbits that did not die spontaneously were sacrificed after a period of 15 to 25 minutes and autopsied immediately. A total of 19 rabbits were used, of which 10 received the antifoaming agent and 9 received control solutions. A gross estimate of the degree of pulmonary edema was made by observing the lungs and tracheobronchial tree. A more quantitative estimate was obtained by determining the ratio of lung weight to body weight. This ratio in normal untreated rabbits was approximately 0.46 but to account for the presence of pulmonary congestion a

ratio above 0.6 was considered indicative of pulmonary edema. Lung:body ratios seemed to correlate well with the degree of observed pulmonary edema. Since the scope of this study was limited to evaluating the effect of antifoaming agent on the development of gross pulmonary edema, ultimate recovery and survival, and the histologic appearance of the lungs were not studied. The findings are summarized in Table II. All of the control animals developed frank pulmonary edema. In contrast 5 out of 10 antifoam treated animals showed evidence of pulmonary edema. Half of the treated group received 5% solutions of DCAA and the other half 10% solutions. It would seem that the antifoaming agent had significantly reduced the incidence of pulmonary edema.

Comment. The usefulness of antifoaming agents in the treatment of pulmonary edema is suggested by the finding that the condition could be prevented in half of the treated animals. In those animals in which pulmonary edema developed in spite of treatment with antifoaming agent, there is always a certain amount of doubt whether adequate amounts of the agent had penetrated deeply enough. It is felt that the availability of a more satisfactory antifoaming agent and a more efficient way of administering it might have increased the proportion of successfully treated animals beyond the reported 50%.

Summary. 1. The collapse of the foam which fills the tracheobronchial tree in certain cases of pulmonary edema would be advantageous in the management of this condition. This might be accomplished by the use of antifoaming agents. 2. A method for measuring antifoaming activity *in vitro* is described and various antifoaming substances are compared by this method in terms of their foam-destroying power. 3. The inhalation of one of these substances, a solution of the silicone DC Antifoam A, in ether, was shown to reduce the incidence of adrenaline induced pulmonary edema in rabbits by 50%.

DCAA: DC Antifoam A, supplied through courtesy of Dow Corning Corp., Midland, Mich.

Aerosol 18 and Aerosol OT, supplied through courtesy of Am. Cyanamid Co., New York City.

TBP: Tributylphosphate, supplied through courtesy of Commercial Solvents Corp., New York City.

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Received May 21, 1952. P.S.E.B.M., 1952, v80.

Effect of Cortisone and Adrenocorticotropic Hormone on Delayed Hypersensitivity to 2,4-Dinitrochlorobenzene in Guinea Pigs.* (19734)

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Cortisone and adrenocorticotropic hormone (ACTH) have been reported effective in suppressing skin reactivity of the delayed type to tuberculin in animals(1-3), and in human beings(4). Varied results have been reported in immediate type of allergic reactions, *e.g.*, anaphylaxis(5-7), Prausnitz-Küstner reactions(8), and Arthus reactions(9). ACTH accelerates the recovery rate of clinical contact dermatitis provided the offending contactant has been withdrawn from the skin (10). No investigations of the locus of the effect of these hormones on delayed reactivity to simple chemical compounds in experimental animals have been reported.

We undertook the present investigations with the purpose of determining: 1) the effect of these hormones in varied doses on guinea pigs previously sensitized to 2,4-dinitrochlorobenzene; and 2) the effect of therapy during the sensitization period on subsequent skin reactivity.

Materials and methods. White and light-skinned guinea pigs weighing 250-350 g and maintained on an *ad libitum* diet of rabbit pellets (Purina) and leafy vegetables were used in all experiments. All animals were sensitized with 2,4-dinitrochlorobenzene using a modification of the method of Landsteiner and Jacobs(11). A 1% solution of 2,4-dinitrochlorobenzene (Eastman) in olive oil was painted on a shaved area of skin at the back of the neck each day for 7 days. After an additional 7 days the animals were tested with the chemical in oil on an area of the back approximately 1 cm square which had been shaved and treated with depilatory cream (Neet). Skin reactions were read at 24 and 48 hours after testing. Reactions were designated arbitrarily as: +++, marked homogeneous erythema; ++, homogeneous erythema; +, erythema; and 0, no reaction.

The hormones (Cortone, Merck, and Acthar, Armour†) were administered intramuscularly in the hip. Cortisone was administered twice daily at 8 A.M., and 3 P.M., and ACTH was given 3 times daily at 8 A.M., 3 P.M., and 10 P.M. All animals were observed frequently for signs of drug toxicity.

Results. Series 1. Three groups of 8 animals which had been previously sensitized and which showed marked skin reactions were used. One group was used as a control, the second received cortisone, 10 mg per day, and the third group received ACTH, 15 mg per day. The animals were treated for 48 hours and the skin was tested. Therapy was continued through the observation period. No difference in skin reactivity was noted between the groups.

Series 2. Groups of 4 animals were treated in this series, the dose of cortisone being increased to 20 mg and 30 mg per day for 2 groups, and the dose of ACTH being increased to 30 mg and 45 mg per day. Again no difference in reactivity was noted between experimental groups.

Series 3. Since the animal tolerated the hormones well, the dose of cortisone was increased to 50 mg per day, and the dose of ACTH to 75 mg per day. A diminution of skin reactivity was not evident. Table I shows extremes in reactions for each group of the first 3 series.

Series 4. Since these hormones proved ineffective in preventing skin reactivity once sensitization had been initiated, it seemed possible that the drugs might in some way affect the course of sensitization. To test this hypothesis 7 groups of 4 animals each were sen-

* This investigation was supported by a grant from the Central Scientific Fund, College of Medicine, State University of Iowa.

† We are indebted to Merck and Co., Rahway, N. J., for Cortone brand of Cortisone acetate and to Armour and Co., Chicago, Ill., for Acthar brand of ACTH used in these experiments.

TABLE I. Effect of Varied Dosage of Drugs on Skin Reactivity in Previously Sensitized Animals.

Treatment	Dosage/day, mg	Skin reactivity 24 hr	Skin reactivity 48 hr
Control	0		
ACTH	15		
Cortisone	10		
ACTH	45	2+ – 3+*	+ – 2+
Cortisone	30		
ACTH	75		
Cortisone	50		

* 3+ = marked homogeneous erythema; 2+ = homogeneous erythema; + = erythema; 0 = no reaction.

TABLE II. Effect of Treatment During Sensitization.

Group	Dosage/day, mg	Skin test 24 hr	Skin test 48 hr
Control	0	2+ – 3+*	+ – 2+
Cortisone	10	2+ – 3+	+ – 2+
	20	3+	2+
	30	2+ – 3+	+ – 2+
ACTH	15	2+ – 3+	+ – 2+
	30	2+ – 3+	+ – 2+
	45	2+ – 3+	+ – 2+

* 3+ = marked homogeneous erythema; 2+ = homogeneous erythema; + = erythema; 0 = no reaction.

sitized as previously, except that doses of 10, 20, and 30 mg per day of cortisone and 15, 30, and 45 mg per day of ACTH were given to 6 groups with the 7th retained as a control. Treatment was continued during the course of the sensitization process (7 days). At the end of this period the sensitizing sites were carefully washed several times with 95% ethyl alcohol in an attempt to remove any residual chemical. After a period of 7 days the animals were skin tested. Extreme results of each group are presented in Table II. No significant differences were observed.

Discussion. These results indicated that cortisone and ACTH in the doses and manner used played no role in diminishing the skin reactivity to 2,4-dinitrochlorobenzene in guinea pigs. Further, it was shown that these animals are not adversely affected by massive doses (200 mg cortisone, 300 mg ACTH per kg body weight per day), when administered over a period of several days. These drugs also proved ineffective under the conditions employed in preventing sensitization with a simple chemical compound.

Summary. Guinea pigs sensitized to 2,4-dinitrochlorobenzene were treated with small to massive doses of ACTH and cortisone without reducing their skin reactivity to topical application of a solution of the drug. Attempts to prevent sensitization by treatment during the sensitization period were negative.

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Received July 23, 1952. P.S.E.B.M., 1952, v80.

Influence of Sex on the Transplantability of Mouse Mammary Tumors. (19735)

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While the relationship between sex and the development of certain spontaneous tumors in mice has been established definitely, there are comparatively few examples of a sex difference in susceptibility to transplanted tumors. Klein(1) cites several of these examples and further demonstrates that for the same tumor certain strains of rats fail to exhibit a sex difference, whereas another strain does exhibit a significant sex difference.

Experiments performed in this laboratory show that transplantable mouse adenocarcinomas always produce 100% takes in either sex, so that in routine transplantation by tumor fragment no sex difference has the opportunity to become apparent because a sufficient number of viable tumor cells is always inoculated to produce 100% takes. However, we have been able to control the percentage of takes by the inoculation of known quantities of viable tumor cells(2-4); and accordingly we have produced takes varying from 0 to 100%, depending on the number of viable cells inoculated. Therefore, if the percentage of takes can be considered a criterion of sex influence, this influence has the opportunity of manifesting itself where the expectancy of takes is less than 100%.

This study concerns observations which show that under certain specific conditions there is a distinct strain difference in the behavior of two transplantable mammary adenocarcinomas in male and female mice.

Materials and methods. The transplantable mouse mammary adenocarcinomas of the "dba" and Marsh-Simpson strains were used in our experiments. The method of preparing the tumors for suspension and counting the viable tumor cells was essentially that of Schreck(5). A subcutaneous tumor 7 to 10 days old, free of necrotic material, was removed aseptically from the mouse and placed in a sterile mortar. The tumor tissue was cut into small pieces with a scissors and then ground thoroughly in the mortar and made into a suspension by the addition of Tyrode's solution. After 5 to 10 minutes of grinding, the suspension was filtered twice through a double thickness of handkerchief linen. To 0.1 cc of the suspension were added 4.9 cc of erythrosin stain dissolved in Tyrode's solution (1-1000) devoid of glucose. The solution was shaken for a 5-minute period and examined in a Neubauer hemocytometer. The appearance of the suspension under the microscope was that of individual cells with few or no clumps. Some cells were stained pink and others were unstained. According to Schreck, the unstained cells are viable cells and were so counted.

Male and female mice of the dba and Marsh strains, about 6 weeks old, were inoculated subcutaneously with 0.05 cc of cell suspensions made from dbrB and Marsh-Simpson tumors, respectively. The mice were palpated semiweekly for the appearance of tumors. When the tumors reached a size of approximately 0.2 cc, the animals were sacrificed and the tumors examined microscopically. Periodically there was a macroscopic

TABLE I. Percentage Takes in Males and Females of the Marsh-Simpson Strain of an Inoculated Transplantable Mammary Adenocarcinoma, Marsh.

	Males			Females		
	Total	Takes		Total	Takes	
Exp. No.	mice	No.	%	mice	No.	%
177	23	8	34.8	21	10	47.6
178	21	19	90.5	19	12	63.2
181	10	9	90	9	8	89
182 C	22	13	59	20	15	75
182	30	7	23.3	19	5	26.3
186	33	6	18.2	26	3	11.5
197	25	16	64	23	20	87
210	34	30	88.2	25	22	88
212	5	4	80	14	11	78.5
219	20	11	55	16	6	37.5
221	30	24	80	10	9	90
222	22	16	72.7	13	10	77
223	8	4	50	29	13	44.8
225	28	10	35.7	14	10	71.5
	311	177	57±1.9	258	154	59.7 ± 2.06

TABLE II. Percentage Takes in Males and Females of the dba Strain of an Inoculated Transplantable Mammary Adenocarcinoma, dbrB.

Exp. No.	Males			Females		
	Total mice	Takes No.	Takes %	Total mice	Takes No.	Takes %
184	14	12	86	17	11	65
190	15	7	47	21	12	57
193	47	37	79	6	5	83
196	5	1	20	27	7	26
202	9	5	56	18	6	33
	90	62	69±3.3	89	41	46 ±3.57

examination of the gross tumor.

Results. A study of our data was carried out where there were less than 100% takes in the 2 strains of mice, *i.e.*, the Marsh and the dba strains. The data for 311 males and 258 females of the Marsh strain are shown in Table I. It is evident from this table that there is no consistent difference between the sexes in the individual experiments and no significant difference between the percentages when all animals are considered collectively.

On the other hand, the data of 90 males and 89 females of the dba strain, although showing no consistent sex influence for the individual experiments, do show a significant difference when considered collectively as shown in Table II.

Discussion. Thus, the growth and development of the dbrB tumor in the dba strain appeared to be related to the sex of the host, whereas no significant sex difference in the number of takes was observed in the Marsh strain when inoculated with a Marsh-Simpson tumor suspension. Experiments are in progress to examine further the possibility that the difference in tumor-take incidence observed for these adenocarcinomas might be explainable on an endocrine basis. From these observations, probably a sex difference in susceptibility to transplanted tumors may be present in some strains and absent in others.

Summary. A significant sex difference in incidence of tumor takes appeared when a known quantity of viable mammary adenocarcinoma cells in a dbrB tumor cell suspension was inoculated subcutaneously into dba mice, where males were more susceptible than females under similar conditions. This sex difference was not noted when a Marsh-Simpson mammary adenocarcinoma tumor cell suspension was inoculated into Marsh mice.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Concentration of Lipid Phosphorus in Tumor and Carcass of Rats Growing Walker Carcinoma 256.* (19736)

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The progressive growth of Walker carcinoma 256 in albino rats has been shown(1-3), to cause a loss of lipid from the carcass, which loss of lipid, rather than failure of appetite, accounts for the caloric deficit produced in the animals(4). In view of the lipemia characteristic of rats growing Walker carcinoma 256 (2,3), and the high concentration of phospholipids in this tumor, a direct comparison of the phospholipid concentration in the tumor and the carcass of the host rat, and in the carcass of the host rat and of suitable control

* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service; and by a grant from the Fluid Research Fund of the University of Rochester School of Medicine and Dentistry.

TABLE I. Concentration of Lipid Phosphorus in Walker Carcinoma 256 and in Normal, Control and Host Rats.

Group	No. rats	Lipid phosphorus, g per 100 g — % dry tissue (avg)	Stand. dev.	% fat-free dry tissue (avg)	Stand. dev.
I Young rats					
a—Normal	11	.156	.010	.210	.011
b—Rats plus tumor	11	.166	.012	.216	.015
II Young rats					
a—Normal	12	.124	.020	.190	.024
b—Rats plus tumor	5	.173	.022	.196	.022
III Adult rats					
a—Controls; non-tumor rats killed when tumors started to grow in rats of c—	7	.089	.003	.137	.012
b—Controls; non-tumor rats pair-fed to tumor rats of c—	7	.092	.010	.134	.018
c—Tumor rats	7				
Carcass		.122	.018	.137	.023
Tumor		.195	.036	.224	.043
Carcass plus tumor		.135	.018	.153	.023
IV Adult rats					
a—Controls; non-tumor rats killed when tumors started to grow in rats of c—	9	.095	.007	.153	.006
b—Controls; non-tumor rats killed with tumor rats of c—	6	.084	.007	.143	.010
c—Tumor rats	8				
Carcass		.106	.009	.156	.013
Tumor		.276	.039	.332	.047
Carcass plus tumor		.131	.016	.186	.020

rats is needed. This paper presents such a comparison for lipid phosphorus and for an alkali-resistant phospholipid, both of which are found to be concentrated in the tumor.

Materials and methods. Male albino rats and Walker carcinoma 256 grown subcutaneously, were used throughout the experiments. The rats of Groups I and II (Table I) were young; their body weights at the time of tumor transplantation averaged 74 g. The animals of Groups III and IV were adult rats from Dr. G. B. Mider's laboratory; the average body weights were 190 g and 230 g, respectively, at the time of tumor transplantation.

The rats of Groups I and II received *ad lib.* Diet 262 containing 21.3% coconut oil (5). In Group III, the rats without tumor were pair-fed with the tumor rats which ate *ad lib.* Data for the rats of this group, for the technic of pair-feeding, and for the diet, have been described previously(4). The loss of appetite which occurs during tumor growth was counteracted by force-feeding the animals of Group IV with the high-fat diet of Ingle (6) modified by the substitution of lactalbumin for casein, and the addition of 1 g of choline per kg of diet. Each rat in the group received 20 g of diet per day (50 cal per day) by stomach tube.

The rats of Group I were killed by diethyl ether 2 weeks after tumor transplantation when the tumors constituted from 0.26 to 8.8% of the total body weight. Group II was killed by diethyl ether 3 weeks or more after transplantation, when the tumors comprised from 22 to 52% of the total body weight. The whole rat plus weighed tumor was ground and dried in the frozen state (lyophilized)(3). The rats of Group IV were decapitated and

the blood collected in saturated citrate. Nine animals without tumor were killed 7 days after transplantation of tumor in the experimental group when the tumor had started to grow. Six rats without tumor were killed when the rats with tumor were killed, at 4 and 5 weeks after transplantation; at this time the tumors were 20% of the body weight, except for one which was only 7%. Lyophilized dried carcass and tumor tissues were prepared as described(3).

The methods used for extraction and determination of the total lipid of the tissues(3) and of the blood plasma(2) have been described previously. In this study the concentration of lipid phosphorus rather than of phospholipid was determined in order to eliminate the use of an oxidative factor for a mixture of phospholipids, since a single factor might be considerably in error in the case of tumor where the phospholipids may differ qualitatively from those in the host rat. Since chloroform-methanol was used for extraction in addition to alcohol-ether, our values represent virtually all the lipid phosphorus of the tissues; (the phospholipids of both wet and dry tissues are incompletely extracted by alcohol-ether alone)(7,8). Lipid phosphorus was determined in duplicate on the solutions of total lipid by the method of Kuttner and Lichtenstein(9). On samples from Group IV, an alkali-resistant phospholipid was isolated from the fatty acid fraction as the lead salt(10) and the amount determined oxidatively.

Results. The values for lipid phosphorus as percentage of the dry tissue and of the fat-free dry tissue, respectively, are given in Table I. That lipid phosphorus concentrates in Walker carcinoma 256, is evident from the results for the adult rats of Groups III and IV where tumor and carcass were analyzed separately. The values for tumor are significantly higher ($P = <.01$ in both groups) than those for carcass of the rat with tumor, whether they are expressed as percentage of dry tissue or of fat-free dry tissue. The difference between tumor and carcass was not the result of a decrease in the lipid phosphorus concentration in the carcass, since the values for carcass are the same or slightly higher than the values for rats killed either when the tumor started to grow or when the tumor rats were killed. The greater concentration of lipid phosphorus in the tumor is also shown by the fact that values are significantly higher ($P = <.01$) in the combined carcass plus tumor than in the non-tumor animals of either of the control groups. The tumor-bearing rats given 50 cal per day of a high-fat diet by gavage (Group IV) had a significantly greater ($P = <.01$) concentration of lipid phosphorus in their tumors than did similar rats which consumed diet *ad lib.* (Group IIIc).

Lipid phosphorus was also concentrated in the tumors of the young rats of Groups I and II since values for the rat plus tumor (percentage dry tissue) are significantly higher (Group I—$P = >.02, <.05$; Group II—$P = <.01$) than values for the normal rats. It is interesting to note that the values for lipid phosphorus of the normal young rats of these groups are higher than the values for the normal adult rats of Groups III and IV.

Although little is known regarding the comparative concentration in tumor and rat of the different kinds of phospholipid which contribute to the high lipid phosphorus concentration of Walker 256, the concentration of a phospholipid fraction which is highly resistant to alkali has been determined for the animals of Group IV and is presented in Table II. The concentration was significantly greater ($P = <.01$) in the tumor than in the carcass of the tumor rat, or in the rats of either of the control groups.

Discussion. The high concentration of phospholipid in tumors as compared with various other tissues of the body has been known for almost half a century. The magnitude of the concentration has now been determined by this comparison between the tumor and the rest of the rat's body. The greater concentration of lipid phosphorus in the tumor than in the carcass was not caused by loss of appetite during tumor growth; the rat plus tumor had a greater concentration of lipid phosphorus than normal rats that received the same diet *ad lib.*, or the same amount and kind of diet by pair-feeding or by gavage, as the respective tumor-bearing rats. The high concentration of lipid phosphorus (phospholipids)

TABLE II. Concentration of Alkali-Resistant Phospholipid in Walker Carcinoma 256 and in Control and Host Rats.

Group	No. rats	Phospholipid, g per 100 g: % dry tissue (avg)	Stand. dev.	% fat-free dry tissue (avg)	Stand. dev.
IV Adult rats					
a—Controls; non-tumor rats killed when tumors started to grow in rats of c—	9	.097	.043	.156	.022
b—Controls; non-tumor rats killed with tumor rats of c—	6	.097	.025	.166	.053
c—Tumor rats	8				
Carcass		.120	.035	.178	.054
Tumor		.373	.052	.448	.065

in the tumor is probably concerned with the enhanced metabolism of fat which may account for the caloric deficit of the animal, and which is undoubtedly related to the lipemia that accompanies growth of this tumor. Evidence for this assumption is not entirely lacking.

The greater concentration of lipid phosphorus in the tumors of rats force-fed a high-fat diet (Group IVc) may have resulted from the necessity for metabolizing excessive amounts of fat. The dietary procedure produced a marked lipemia in the 8 tumor-bearing rats; 7 of them had values for total lipid in the blood plasma ranging from 552 to 1372 mg %, while one had a value of 5350 mg %. The 15 non-tumor rats of this group averaged 423 mg % with a standard deviation of 59. In addition to the excessive lipemia caused by the forced feeding, the concentration of total lipid in the tumors increased by about 30% as compared with the concentration in the tumors of Group III. This increase in total lipid is fully accounted for by increased phospholipid as estimated from the increase in concentration of lipid phosphorus.

When expressed as percentage of the fat-free dry tissue, the increased concentration of lipid phosphorus in rat plus tumor was apparent in adult rats but not in young rats even in those of Group II where tumors comprised over 20% of the total body weight. Tumor tissue is a young tissue with a high lipid phosphorus concentration similar in magnitude to that of young rats. In the tumor the high value for lipid phosphorus was maintained even when the tumor was grown on adult rats of lower lipid phosphorus concentration. The greater growth potential of this tumor in young than in adult rats might conceivably be correlated with the respective lipid phosphorus concentrations of the rats.

The concentrations of most of the component phospholipids which account for the greater concentration of lipid phosphorus in the tumor remain to be determined. However, we have shown that the tumor is rich in a phospholipid which is stable to alkali. Similar (unpublished) data for the concentration of this material were obtained in tumor rats that ate *ad lib.* 2 diets(3) of lower fat content. From the stability toward alkali, the nitrogen and phosphorus contents, the solubility in various organic solvents, the occurrence, and the general behavior of the substance, we assume tentatively that it is a crude acetal phospholipid.

The maintenance by the rat growing Walker carcinoma 256 of a lipid phosphorus concentration, or of the concentration of a particular phospholipid in his tumor, far in excess of that in his carcass, may contribute in large measure to the stress which causes many of the adverse effects of the tumor on the host rat.

Summary. In rats growing Walker carcinoma 256, 1) the concentration of lipid phosphorus was greater in the tumor than in the carcass; 2) the concentration of lipid phosphorus was greater in the rat plus tumor than in pair-fed or force-fed rats without tumor that consumed the same amount and kind of diet as the tumor rats; 3) the concen-

trations of lipid phosphorus in young rats and in the tumor were similar, and greater than in adult rats; 4) the concentration of an alkali-resistant phospholipid was greater in the tumor than in the carcass.

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Received July 23, 1952. P.S.E.B.M., 1952, v80.

Multiple Effects of Fluoroacetate on Pyruvate Metabolism *in vitro*. (19737)

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The injection of fluoroacetate into rats has been shown to result in the accumulation of large amounts of citrate in certain organs (1-5). No accumulation of citrate was found (3) in livers of fluoroacetate-treated rats; these rats were of the same sex and strain as those whose livers had previously been shown to produce large amounts of citrate *in vitro* (6).† A possible explanation for this discrepancy was furnished by studies(7,8) showing that an interruption in the Krebs cycle in the case of liver results in the diversion of C_2 fragments away from the Krebs cycle and into acetoacetate formation, and it might have been assumed that the results could be explained solely on this basis. However, when *in vitro* studies were carried out to test this simple hypothesis, new results were obtained, some of which appear to be unexplainable in terms of the inhibition of citrate removal alone.

* Public Health Service Postdoctoral Research Fellow of the National Cancer Institute.

† The rats used by Potter and Busch(3) were normal males. Subsequent work in this laboratory and in that of Dr. K. P. DuBois makes it clear that hormonal and other physiological factors can alter the capacity of the liver to accumulate citrate following the injection of fluoroacetate. Fed female rats accumulated citrate in their livers following the injection of fluoroacetate, and male rats could be shown to do likewise under special conditions.

Experimental. Chemicals. Sodium monofluoroacetate was supplied by Dr. K. P. DuBois of the University of Chicago Toxicity Laboratory; adenosine triphosphate (ATP) was prepared by Dr. G. A. LePage and associates.

Homogenates. Male rats (weighing 200-300 g) obtained from the Holtzmann-Rolfsmeyer Rat Co. were used in this study. After the rats were decapitated, the kidneys were quickly removed and placed in ice-cold isotonic KCl solution; after weighing, they were placed in glass homogenizers(9) containing 9 volumes of ice-cold isotonic KCl solution and homogenized in the cold; the same procedure was followed with liver homogenates, but the homogenizers contained 5¼ volumes of KCl solution to give a concentration of 16% liver by weight.

Flask contents. a) *Reaction mixtures.* Each Warburg flask contained 1.0 ml of a basal medium composed of 0.01 M $MgCl_2$, 0.2 M KCl, and 0.01 M K phosphate buffer adjusted to pH 7.2(6) as well as 30 μM potassium pyruvate, 3 μM potassium ATP and sufficient 0.2 M or 0.02 M sodium monofluoroacetate‡ to produce the desired concentration. All substrates were added as potassium salts neutralized to pH 7.2-7.4. In all experiments other than those involving phosphate studies, an additional 0.1 ml of 0.1

M K phosphate was added. Center wells contained 0.2 ml of 2.5 M NaOH and a wick of filter paper. Cold homogenates were added to the cold reaction mixtures and the vessels were equilibrated 10 minutes before manometric readings were begun. The gas phase was air and the temperature was 38°C. b) *Homogenate systems.* Three homogenate systems were employed for the purpose of studying the effects of fluoroacetate on pyruvate oxidation. In addition to the basal medium, pyruvate and ATP noted above, the *liver-malonate* system contained 80 mg of liver tissue and 12 μM of potassium malonate; the *liver-fumarate* system contained 80 mg of liver tissue and 40 μM of potassium fumarate, while the *kidney-fumarate* system contained 40 mg of kidney tissue and 40 μM of potassium fumarate. Each of these reaction systems responds differently to fluoroacetate as will be shown below. The concentration of fumarate was selected following the demonstration that increasing the concentration of fumarate increased formation of citrate and decreased formation of acetoacetate by liver homogenates; at 40 μM of fumarate, both of these effects had reached plateau levels(8). Oxygen uptake in 60 minutes was proportional to tissue at concentrations up to 90 mg wet weight of liver per Warburg flask and up to 40 mg of kidney per flask; in general, kidney homogenates were much more labile than liver homogenates.

Determinations. Reactions were stopped by the addition of 0.3 ml of 50% perchloric acid to the main chamber. The protein-free supernatant fluid obtained on centrifugation of the contents of the flask was used for the analyses. Citrate(10), acetoacetate,§ α-ketoglutarate and pyruvate(11), and inorganic phosphate(12) were determined by standard methods.

Results. Comparative studies on kidney and liver homogenates. The data obtained with each of the 3 homogenate systems are presented in Fig. 1-4, and are plotted together for purposes of comparison. The following sections consider each of these systems separately.

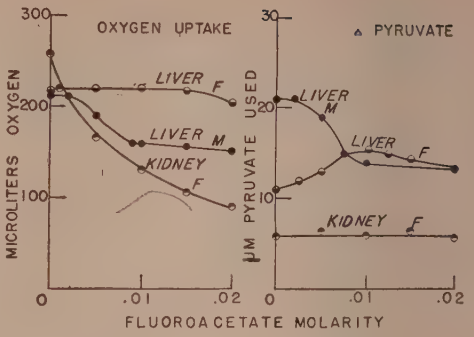

Fig. 1

Fig. 2

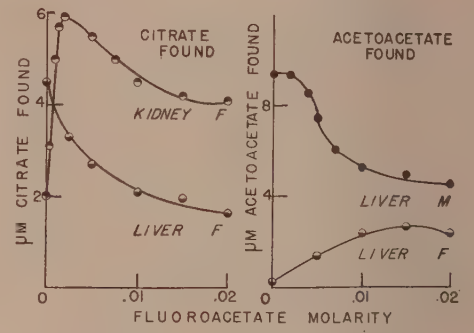

Fig. 3

Fig. 4

FIG. 1–4. Effects of fluoroacetate concentration on oxygen uptake, pyruvate utilization, citrate formation and acetoacetate formation by the liver-fumarate system (Liver F), liver-malonate system (Liver M) and kidney-fumarate system (Kidney F) described in the text. Time of incubation: 60 min.

Effect of fluoroacetate on the kidney-fumarate system. The sole pathway of pyruvate oxidation in this system is the citric acid cycle(13). At *low* concentrations of fluoroacetate a maximal accumulation of citrate was found (Fig. 3). However, as the concentration of fluoroacetate was increased, the block of oxidation was established earlier and/or more completely (Fig. 1) and this progressive depression of oxidation was paralleled by depression of citrate formation. While initial oxidation was depressed less in this system

‡ Trifluoroacetate inhibited neither the kidney-fumarate nor the liver-malonate system. Recrystallized difluoroacetate did not inhibit the liver-malonate system and the slight inhibition of the kidney-fumarate system at high concentrations may have been due to monofluoroacetate contaminating the product.

§Lehninger, A. L., Personal communication, see (8).

than oxidation at later time intervals, increasing concentration of fluoroacetate also depressed the initial oxidation.‖

The block of citrate oxidation is probably limited to a tricarboxylic acid step and at low levels of fluoroacetate it appears that the other reactions are not blocked, since a micromole of citrate is formed for each micromole of fumarate added to kidney homogenates inhibited *in vitro* (Fig. 5).¶

Effect of fluoroacetate on the liver-malonate system. The liver-malonate system also has one pathway for pyruvate oxidation, but in this case oxidative decarboxylation of pyruvate is followed by the condensation of the "C_2" fragments to acetoacetate. In this system, the depression of oxygen uptake (Fig. 1) was associated with a simultaneous diminution of pyruvate utilization (Fig. 2) and acetoacetate formation (Fig. 4) but these effects were not noted until *higher* concentrations of fluoroacetate were present as compared to the kidney-fumarate system. Moreover, the oxidation was increasingly depressed as the incubation progressed. The rate of oxidation of the blocked system here, however, was the same as that of the control until a time (30 minutes of incubation) when its activity suddenly decreased; oxidation by the control continued at a linear rate for at least 60 minutes. This delayed response accounts for the failure of high levels of fluoroacetate to produce more than a limited response in the measurements undertaken, and suggests that the action of the inhibitor must have been indirect just as in the kidney-fumarate system. It is unlikely, however, that the eventual decline in rate can be attributed to the formation of fluorocitrate since in this system essentially no oxidation takes place via the citric acid cycle, *i.e.*, no more than 0.25 μM of citrate were found in any of these flasks, and moreover there is no basis for explaining the results in terms of a block in the citric acid cycle, which was already blocked by malonate in this system. The time studies revealed that the inhibition of the liver-malonate system was not competitive; moreover, the inhibition was not prevented by addition of as much as 0.03 M $MgCl_2$(14).

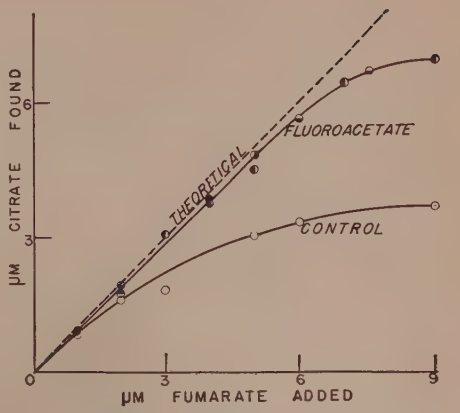

FIG. 5. Conversion of fumarate to citrate by kidney homogenates poisoned *in vitro* with fluoroacetate compared to controls. Additions as in kidney-fumarate system described in the text except for quantity of fumarate added. Flasks with fluoroacetate contained 6 μM.

Effect of fluoroacetate on the liver-fumarate system. The liver-fumarate system can oxidize pyruvate by both of the above mentioned pathways; these 2 pathways are in fact competitive, with citrate formation preferential when fumarate is available(8). Any differential effect of fluoroacetate would thus be revealed in this reaction as a shift away from

‖ Maintenance of bound phosphate was observed in the liver-fumarate system; bound phosphate of the kidney-fumarate system diminished as oxygen uptake decreased, in control flasks as well as in flasks containing fluoroacetate. Bound phosphate decreased rapidly even in control flasks of the liver-malonate system although oxidation proceeded at a linear rate, and at the end of 60 minutes of incubation, almost all the high energy phosphate had been released from ATP.

¶ In these experiments, oxygen uptake studies indicated that extra pyruvate disappearing was largely oxidized to completion and hence, fumarate must have cycled but was eventually trapped as citrate; for example, in a 60 minute incubation period, with 4 μM of fumarate and 30 μM of pyruvate as substrates, 6.7 μM of pyruvate disappeared, of which 3.8 μM entered into citrate formation; total oxygen uptake was 8.5 μM in the measured 50 minute period after the 10 minute equilibration period. The consumption of considerably more oxygen than the 1.9 μM necessary for conversion of pyruvate to citrate indicated that some fumarate proceeded around the cycle more than once but was eventually stopped only at the citrate level, suggesting that the other parts of the cycle were unaffected.

the normal ratio of citrate to acetoacetate. When the fluoroacetate concentration was increased, there was a diminution in citrate formation (Fig. 3) paralleled by a compensatory rise in acetoacetate formation such that pyruvate utilization actually increased (Fig. 2), while oxygen uptake continued unaffected (Fig. 1).

Discussion. The results indicate that fluoroacetate or its derivatives may inhibit 2 enzymatic steps in addition to aconitase, which has been studied in considerable detail by Peters and associates(15). The liver homogenate illustrates one of these inhibitions by decreasing the metabolism of pyruvate via the citric acid cycle and shifting to the alternative pathway of acetoacetate production. It seems likely that this result is due to a direct inhibition of an "acetokinase"(16) rather than to a shift resulting from the unavailability of C_4 acids, a mechanism studied by Lehninger(7), and by Recknagel and Potter(8), inasmuch as a considerable backlog of fumarate was present when fluoroacetate was studied. Differences in the K_I and K_M for these acetokinases and fluoroacetate or inhibitors formed from it must result in the metabolic shifts noted, and it appears that the acetokinase leading into the citric acid cycle is affected to a greater extent than the other acetokinases in liver. The decreased utilization of C_2 fragments for citric acid formation is reflected in an increased availability for acetoacetate formation *in vitro* and by the increased acetylation of foreign amines in intact rabbits treated with fluoroacetate(17). That the formation of acetoacetate can also be inhibited is shown by the data obtained with higher levels of fluoroacetate. The finding of Hagan *et al.*(18) of significantly less fluoroacetate in livers of poisoned rats than in other organs tested may result from utilization of "fluoroacetoacetate" formation as a means of detoxification of fluoroacetate by the liver.

The *in vitro* studies reported here suggest that the pharmacological effects of fluoroacetate may involve the production of more than one type of "biochemical lesion," while species differences may well reflect differing ratios of the several enzymes involved.

Summary. 1. Citrate formation is increased and oxidation is diminished by addition of fluoroacetate to kidney homogenates; under the same conditions, citrate formation is decreased and acetoacetate formation is increased while oxidation is unchanged in liver homogenates. 2. At higher concentrations of fluoroacetate, acetoacetate formation is also inhibited in liver homogenates.

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Received July 23, 1952. P.S.E.B.M., 1952, v80.

Comparative Amoebacidal Activity of Some Compounds Related to Emetine.*† (19738)

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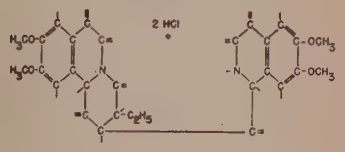
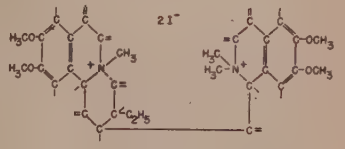
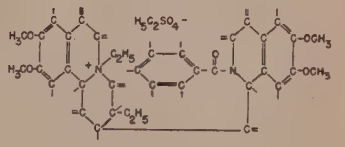
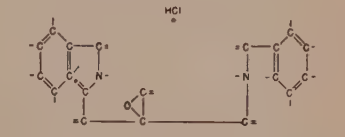
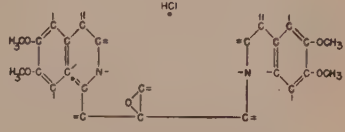
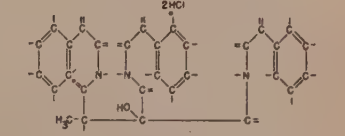
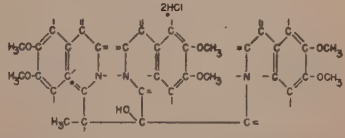
Although emetine, in its various pharmaceutical preparations, was early used in the treatment of amoebiasis(1), has retained its position among the most modern drugs in the therapy of this disease. Although its usefulness as an amoebacidal agent is seriously limited by its cumulative properties, cardiotoxic effects, and narrow margin of safety(2), many workers believe that somewhere in the structure of emetine(3-5) lies the answer to a useful amoebacidal drug.

Several attempts have been made to obtain compounds structurally related to the formula of emetine, eliminating the undesirable side-effects while retaining or enhancing the amoebacidal action of the alkaloid. Pioneer research was done by Pyman and his coworkers (6). Goodson and his associates(7-9), and Sugasawa's group(10) have helped clarify the pharmacophore of amoebacidal agents; recent contributions were made by Osbond(11). A detailed treatment of this topic is available elsewhere(12).

The present report originates from an investigation of a synthesis of amoebacidal agents structurally related to emetine, directed by Dr. George L. Webster. During the chemical study a series of phenylalkylamides of phenylalkylaminoisovaleric acids was prepared(13). A different approach to the problem is represented by quaternary emetine derivatives, whose chemistry and pharmacology have been reported(14).

The object of the present report was to de-

TABLE I. Structural Formulae of Emetine and Related Compounds.

Designation	Formula*
Emetine · 2HCl	
E–1	
E–2	
V–1	
V–2	
V–3	
V–4	

* Structure as established by Pailer and Porschinski(3), Battersby and Openshaw(4), and Evstigneeva, *et al.*(5).

* The authors take pleasure in acknowledging the valuable advice of Drs. G. L. Webster and C. C. Pfeiffer, University of Illinois, Chicago.

† The biological phases of this work were supported in part by a research grant (to W. B.) from the Division of Research Grants, U. S. Public Health Service. The technical assistance of Mr. Duncan A. McCarthy is acknowledged.

‡ Present address: Monsanto Chemical Company, St. Louis 4, Mo.

TABLE II. Relative Ranking of Selected Compounds Related to Emetine, in Terms of Amoebacidal and Bactericidal Activity, and General Toxicity.

Chemical agent	Amoebacidal endpoint (μg/ml)	Bacterial growth ($\times 10^6$/ml)	pH at end of test	Approx. LD_{50} (mg/kg) in mice, i.v.
V–3	200	485	7.8	60–70
V–1	500	120	7.8	120–125
V–4	2000	130	7.7	160–180
V–2	4000	90	7.2	160–200
E–2	500	130	7.7	16*
E–1	5000	100	7.8	9*
Emetine·2HCl	1	540	7.8	16
Control		400	7.5	

* Lasslo and Kimura (14).

termine the relative activity *in vitro* of these compounds, and to draw any pertinent conclusions relating chemical constitution to amoebacidal activity.

Materials and methods. The compounds in question were tested for amoebacidal activity *in vitro,* in comparison to emetine hydrochloride (Table I); the isovaleric acid derivatives are drawn so as to emphasize their constitutional relationship to emetine. A known antibacterial agent, aureomycin hydrochloride, was added to the series for comparison with any antibacterial compounds, in the event of uncertainty about direct versus indirect amoebacidal action of such agents.

Aqueous stock solutions were prepared of all compounds, or were weighed directly into culture tubes in those cases requiring high concentrations. The test medium was liver-egg yolk infusion (pH 7.4) plus rice starch (15). Tests were conducted with the *UC* strain of *Entamoeba histolytica* growing with 2 strains of *Escherichia coli.* The definitive experiments involved 5 ml volumes in 16 x 150 mm Pyrex tubes: 4.25 ml sterile medium + 0.5 ml drug in distilled water + 0.25 ml inoculum of pooled amoebae and bacteria. Amoebic inocula exceeded 100,000 per tube. Experimental runs extended 48 hours, record being made of amoebacidal endpoints along with bacterial populations, pH, and oxidation-reduction (O-R) potentials at those critical concentrations. In all, 9 series of experiments were conducted in determining specific endpoints.

To determine the advisability of possible further experiments *in vivo,* the comparative acute toxicity of these compounds was determined in cooperation with Miss Elizabeth H. Jenney of the Department of Pharmacology, University of Illinois. The relative acute toxicity was determined on the Harlan strain of Swiss mice, by intravenous injection into the tail vein of the animals (avg wt. 20-25 g), of 0.1-0.2 ml of solution during a period of 20-25 seconds. Owing to the limited quantities available of each compound, the small number of animals used (15 to 20 per compound) permitted only approximate estimation of LD_{50} values.

Results. The activities of the several compounds are summarized in Table II. It is clear that emetine ranks alone in its amoebacidal activity, in agreement with most *in vitro* findings (16,17). The relative standings within the 2 groups of compounds are important. *V-3* and *E-2* were distinctly most active in their respective groups. It may be noted that vioform, an accepted therapeutic agent, yielded endpoints of 100-200 μg per ml under similar testing conditions (18). Under the aerobic conditions of incubation used in fixing endpoints, the pH values remained at low alkaline levels throughout the experiments. Some antibacterial effect was exhibited above levels of 200 μg per ml, but our experience over a period of several years had indicated that minimal bacterial populations of the order of 10^7-10^8 per ml were suitable for amoebic growth.

The case of aureomycin, on the other hand, discloses the difficulty of interpretation raised by strongly antibacterial agents. At the apparent amoebacidal endpoint of 17 μg per ml under aerobic conditions, and with pH values similar to those found with other agents, bac-

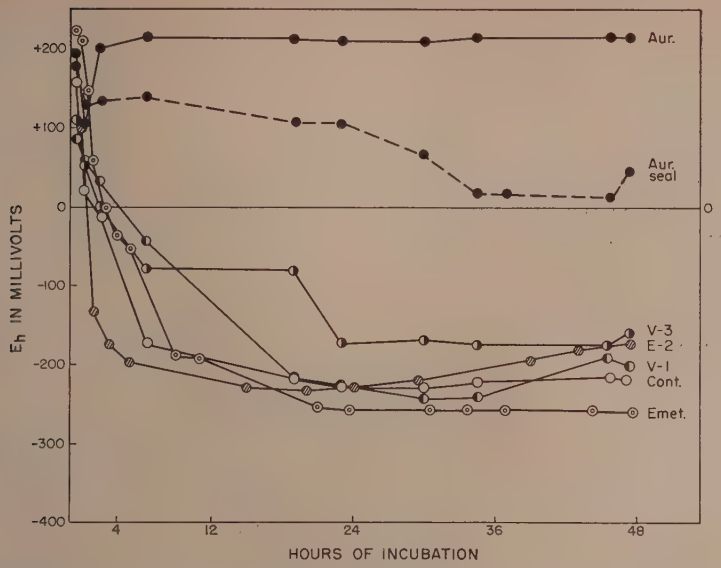


FIG. 1. Oxidation-reduction potentials (E_h) in cultures of *Entamoeba histolytica* containing amoebacidal concentrations of the following agents (experiments conducted in liver-egg yolk infusion under aerobic incubation unless otherwise specified): Cont.—control; Emet.—emetine 1 μg/ml; Aur.—aureomycin 100 μg/ml; Aur. seal—same, with petrolatum seal; E-2—500 μg/ml; V-1—500 μg/ml; V-3—500 μg/ml.

terial populations reached less than one-hundredth of the average of other agents tested. Under these circumstances it becomes impossible to separate direct from indirect action of such an agent against the amoebae, since the amoebae require the presence of metabolizing bacteria in cultures of this kind. A further complication arises from the difference in susceptibility of different floras to antibacterial agents. For example, we have shown (summarized in 17) that the *NRS* strain of *E. histolytica*, in the presence of different floras from the one used here, survived in concentrations of aureomycin as great as 200 μg per ml.

It is well known that O-R potentials, as expressed by E_h, provide a good measure of the reducing activity of microbial cultures(19), and that low potentials (*i.e.*, negative values of the order of —100 to —300 mv) form one requirement for good growth of amoebic cultures(20,21). Time-potential curves were traced for amoebacidal concentrations of emetine and the 3 most active compounds of the series (Fig. 1). These data are in agreement with those for bacterial survival, since they indicate that at amoebacidal endpoints the O-R potentials reach levels compatible with good amoebic growth.

The present experiments afforded an opportunity to note the comparative effects of aerobic and anaerobic incubation upon amoebacidal endpoints. Bradin and Hansen(21) have reported that melted-petrolatum seals greatly increase the "apparent amoebacidal endpoint"‖ of cultures containing antibacterial agents. Thus, in their experiments the amoebacidal endpoints for aureomycin in cotton-stoppered and petrolatum-sealed cultures respectively were 2 μg per ml and 50 μg per ml. This protective influence was attributed to preventing entrance of oxygen into a test medium of already lessened reducing activity, and O-R potentials were distinctly more negative under anaerobic incubation. On the other hand, as might be expected with a non-antibacterial agent, in their experiments emetine displayed a constant endpoint (10 μg per ml)

‖ *Amoebacidal endpoint* is that concentration of agent at which no viable amoebae can be recovered, either by direct examination or upon subculture into sterile culture medium.

with both kinds of incubation.

The bearing of this factor on the present results was investigated. In all cases anaerobic incubation caused a shift in amoebacidal endpoint to higher concentrations by a 2- to 5-fold factor. Whereas Bradin and Hansen had reported this effect only with antibacterial agents, we observed the same effect with compounds which were essentially not antibacterial (Table II); this also held true for emetine. A possible explanation of this discrepancy will be offered below. Time-potential curves of anaerobic cultures resembled those of Bradin and Hansen, in disclosing somewhat more negative potentials for emetine-like compounds than of parallel aerobic cultures. This effect was most striking with aureomycin (Fig. 1), where extreme bacterial inhibition exaggerated differences in reducing activity. The endpoint for aureomycin under anaerobic conditions was 100 μg per ml (*i.e.*, about 6 times greater than under the aerobic conditions reported above), despite a surviving flora of only 0.17 x 10^6 per ml (compared to 0.7 x 10^6 per ml at the same concentration under aerobic conditions) and relatively constant pH values between 7.05 and 7.30 in all aureomycin-treated cultures. It seems clear that aureomycin inhibited bacterial metabolism so drastically that bacterial populations were inadequate to maintain the amoebae.

The bacterial flora used in the present experiments were distinctly aerobic;§ thus, petrolatum-sealed control tubes contained only about one-fourth as many bacteria as aerobic controls; this was the case with all agents tested. This created the paradoxical situation of the same condition apparently both inhibiting the bacterial flora and protecting the amoebae. However, one important variable not studied critically by Bradin and Hansen could be shown to operate. In all petrolatum-sealed cultures—excepting the aureomycin series—the pH was lowered by one unit or more, presumably owing at least partly to retention of gaseous metabolites. This effect of pH on reducing the amoebacidal activity of emetine was discovered by Dobell and his colleagues(16), but frequently has been neglected since then.¶ Our data demonstrate that both experimental series of compounds behave like emetine. The indifferent effect of anaerobic incubation in the emetine tests of Bradin and Hansen may have been due to the relatively low pH existing in both kinds of incubation with their test medium and flora, and this in turn would explain the relatively high amoebacidal endpoint reported by them.

Summary and conclusions. 1. Members of 2 different series of compounds were tested against cultures of *E. histolytica* grown with *E. coli*. 2. *E-2* proved the more effective of the 2 quaternary emetine derivatives, exhibiting an amoebacidal endpoint of 500 μg per ml; this was 10 times as effective as *E-1*. 3. Of the series of phenylalkylamides of phenylalkylaminoisovaleric acids, *V-3* and *V-1* proved approximately 10 times more effective than the corresponding methoxy-substituted moieties. *V-3* was the most amoebacidal of all the above compounds, with an amoebacidal endpoint of 200 μg per ml, while the endpoint for *V-1* was 500 μg per ml. 4. The amoebacidal activity of the compounds was shown to be independent of effects upon the associated bacterial flora, as measured both by surviving bacterial populations and oxidation-reduction potentials of the cultures. This activity was definitely influenced, however, by the pH during tests, more alkaline conditions being correlated with greater effectiveness. 5. Parallel tests with aureomycin, included to reveal effects of a distinctly antibacterial agent, supported the conclusion that, unlike aureomycin, the present series of compounds had acted directly against the amoebae.

Some concluding observations on structure-activity relationship. Quaternization of both nitrogens in the emetine molecule (*E-1*)

§ On the other hand, the flora used by Bradin and Hansen consisted of a single *Clostridium*-like anaerobe (organism *t*) with relatively fastidious nutritional requirements.

¶ At present it is not possible to separate the biological and chemical phases of this effect of pH. The influence of pH on emetine itself in media is not entirely clear. On a physico-chemical level the hypothesis might be advanced that the solvation, and therefore the relative effectiveness, of emetine varies directly with the hydrogen-ion concentration of its medium,

yielded weak amoebacidal action. *E-2,* with only one quaternized nitrogen, proved a considerably stronger amoebacidal agent. It might be concluded that quaternization of the nitrogen atoms in the emetine molecule decreases amoebacidal activity, the number of quaternized nitrogen atoms being inversely proportional to amoebacidal action. Correlation of the pharmacologic studies on the quaternary emetine derivatives(14) with the amoebacidal tests indicates that, although quaternization of both nitrogens produced a compound lacking the cumulative and cardiotoxic effects of the original alkaloid, this derivative exhibited a strong curare-like action but only weak amoebacidal properties. Under these circumstances, a promising lead might be offered by a partially quaternized emetine derivative, which could reach a therapeutically active level in the host's tissues without causing a curare-like paralysis of the neuromyal junctions of the striated musculature.

In the other series of compounds, our data indicate that 3,4-dimethoxy substitution of phenyl radicals decreased substantially the amoebacidal action (*V-2, V-4*). Furthermore, the phenylalkylamides of the monophenylalkylaminoisovaleric acids (*V-1, V-2*) were less amoebacidal than the corresponding phenylalkylamides of the bis-substituted ones (*V-3, V-4*). It should be noted, however, that the influence of the epoxy group and the corresponding hydroxy function is not known and cannot be determined from this series of compounds alone.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Suppression of Viral Pneumonia in Mice by a Microbial Product (APM)* (19739)

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For several years a systematic search for antiviral agents has been conducted in this laboratory. Two antibiotics were found which possessed antiviral properties, namely, Ehrlichin(1) and viscosin(2). Neither of these preparations appeared to be of sufficient interest to warrant further study and the search was resumed. Filtrates from cultures of various microorganisms recently isolated from soil were tested for antiviral activity against influenza A virus in both embryonated eggs and mice. Of the cultures tested, one, a culture of *Achromobacter sp.* 134,‡ produced a material endowed with a unique combination of biological characteristics. This material was found to suppress the development of pulmonary lesions in mice which resulted from infection with influenza A virus and to reduce the non-transmissible pneumonia induced by the intranasal instillation of large numbers of infectious particles of Newcastle disease virus. Further, attempts to demonstrate virucidal activity against these viruses were unsuccessful and antiviral activity in embryonated eggs was, at best, barely detectible by the usual methods.

Materials and methods. Pertinent information on the various viruses used in these studies and on the procedures followed in the use of embryonated eggs have been described (1,2). Hemagglutination tests were performed in the usual manner(3). Albino mice (Webster strain) weighing 18 to 20 g were infected intranasally under light ether anesthesia with 0.05 ml of appropriately diluted virus as indicated in the text. Parenteral injections of test materials were begun one hour or more after infection or as indicated. The lungs of each mouse were carefully examined and the degree of pulmonary consolidation was scored as described by Horsfall(4). The average weight of the lungs of the various groups of mice was frequently determined as an additional criterion.

Description of Achromobacter sp. No. 134. The bacteria measured approximately 0.7 by 2 to 10 μ and occurred singly or in short chains. The rods were gram-negative, non-acid-fast, non-spore-forming, and were motile by means of peritrichous flagella. The organism failed to grow anaerobically. On nutrient agar young colonies were circular, low convex, white, entire, adherent and measured 1.0 to 1.5 mm in diameter. Older colonies gradually developed radial striations with a lobate edge. A pellicle was usually formed in liquid media. Litmus milk was alkaline and reduced. An acid reaction was produced in media containing glucose, galactose and maltose and inorganic ($(NH_4)_2SO_4$) but not organic nitrogen. Nitrites were formed from nitrates. Production of H_2S was slight or absent. Indol was not formed. Starch and gelatin were hydrolyzed. Citrate was utilized as the sole source of carbon. The optimum temperature was 28°C to 36°C. The culture was isolated from soil.

Preparation of APM. Five ml amounts of an actively growing culture of *Achromobacter sp.* No. 134 were inoculated into each of a series of Blake bottles containing 250 ml of medium composed of 0.25% glucose, 1.0% peptone, 0.5% beef extract, and 0.1% yeast extract in distilled water. After incubation for 48 hours in the horizontal position the

* Paper of the Journal Series, N. J. Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Microbiology. These investigations were supported by a grant-in-aid from the Rutgers Research and Endowment Foundation.

† It is a pleasure to thank Dr. Selman A. Waksman for his interest and encouragement and Dr. Ruth Gordon, Dr. Hubert Lechevalier, Mr. Ralph Gwatkin and Mr. Willard Taber for assistance in various phases of the work.

‡ This culture has been deposited in the culture collection of the Department of Microbiology under the number 134.

TABLE I. Suppressive Effect of APM on Pulmonary Lesions in Mice Infected with Influenza A Virus.

mg APM inj. SC on day: 0	1	2	Result–3rd day after infection* I/T	Lesion score L/M	%	Avg wt lungs, g
0	0	0	21/22	48/105	46	
9	9	9	6/12	4/60	7	
3	3	3	12/12	8/60	13	
1	1	1	6/10	5/50	10	
.3	.3	.3	10/12	10/60	17	
.1	.1	.1	11/12	21/60	35	
			0/6 †			.18
0	0	0	15/15	53/75	71	.33
3	3	3	5/12	7/60	12	.22
0	3	3	12/12	20/60	33	.22
0	0	3	12/12	28/60	47	.24
0	3	0	11/12	19/60	32	.25
3	3	0	10/12	20/60	33	.23
3	0	0	8/12	14/60	23	.24

* Approximately 10000 ID_{50}.

† Uninfected controls.

SC = subcutaneously. I/T = No. of mice infected/total. L/M = Total lesion score/total max score. % = L ÷ M × 100.

Lesion score: ½ = <5% lung tissue consolidated; 1 = 5-25%; 2 = 26-50%; 3 = 51-75%; 4 = 76-100%; 5 = dead mouse with lungs consolidated.

culture was filtered through glass wool to remove the pellicle. The culture filtrates were pooled and acidified with concentrated HCl to pH 3.2 to 3.5. A whitish precipitate was formed which slowly flocculated. This precipitate was allowed to settle by gravity and the supernatant fluid was carefully removed by suction and discarded. The precipitate was then suspended in 10 to 20 volumes of distilled water and dissolved by the addition of 5 N NaOH to pH 8. The material was then reprecipitated 3 times in a similar manner. Before the final precipitation the solution was clarified using the Sharples supercentrifuge. The final (4th) neutralization was performed in a small volume of water which was dried from the frozen state. The average yield was approximately 110 mg of APM per liter of culture. This preparation was provisionally designated APM (acid precipitable material).

Experimental. Experiments with influenza A virus. Data from 2 typical experiments illustrating the suppressive effect of APM on the development of pneumonia in mice infected with influenza A virus are presented in Table I. Groups of 12 or more mice each were infected intranasally with approximately 10,000 ID_{50} of influenza A virus and injections of APM were begun one hour or more after infection as indicated. All mice were sacrificed on the 3rd day after infection and the degree of pulmonary consolidation and average weight of the lungs were recorded. The data indicate that (a) daily injections of APM were the most effective, (b) a delay of 24 hours in the time of treatment or a reduction in the number of injections of APM reduced its effectiveness, (c) a suppressive effect was still demonstrable when only one injection of APM was given 48 hours after infection, and (d) daily injection of as little as 0.3 mg of APM exerted a detectible suppressive effect on the development of pulmonary lesions. It is of interest in this connection to recall that the infective titer of lung tissue has been shown to be maximal 24 hours after infection with low dilutions of influenza A virus(5).

Comparative studies on the relative effectiveness of APM administered by various routes were carried out in similar experiments with influenza A virus. The data obtained indicated that intraperitoneal injection of APM was also effective but was toxic and that oral administration of 4 mg per day of APM was without effect. Intranasal instillation of APM-virus mixtures was also found to be ineffective in the following experiment. One ml amounts of serial decimal dilutions of influenza A virus were mixed *in vitro* with an equal volume of saline alone and saline containing 3 mg of APM per ml, respectively, and incubated for one hour at room temperature before inoculation into groups of 5 mice each by the intranasal route. The mice were observed for a period of 10 days and dead mice and mice still living on the 10th day after inoculation were examined and the degree of typical pulmonary consolidation was recorded. The end points (ID_{50}) of the 2 titrations were identical ($10^{-7.3}$) clearly indicating that APM was not virucidal for influenza A virus.

It was of interest to determine whether APM was more effective when mice were infected with a much smaller quantity of influenza A virus. In the experiment summarized in Table II, groups of 20 mice each were

TABLE II. Effect of APM on Degree of Pulmonary Consolidation in Mice Infected with Sublethal Dose of Influenza A Virus.

APM	Result–10th day after infection* Lesion score I/T	L/M	%
Control	17/20	34/100	34
1 mg SC × 10	5/20	9/100	9
3 mg SC × 10	6/19	6/95	6

* 10 ID_{50}.

TABLE III. Effect of APM on Rate of Death of Mice Infected with 10000 ID_{50} of Influenza A Virus.

APM: dose/day	I/T	Cumulative % mortality on day: 2	3	4	5	6	7
Control	19/20	0	15	45	80	85	95
1 mg SC	20/20	0	0	20	50	95	95
3 "	20/20	0	0	0	25	60	95

TABLE IV. Effect of Culture Filtrate and APM on Influenza A Virus in Eggs.

Type of test	Test material	Result I/T	Result Avg hemagglutinin titer
Contact	Control	10/11	626*
	Culture filtrate	9/11	380
Therapeutic	Control	13/14	731
	Culture filtrate	11/16	270
Protection	Control	14/15	955
	Culture filtrate	12/14	340
Contact	Control	7/7	1473
	2.5 mg APM†	4/4	840
	1.25	7/8	740

* Reciprocal of avg hemagglutinin titer of allantoic fluid collected 48 hr after infection with 10-100 ID_{50}.

† Sterilized with ethylene oxide.

Contact test = test material mixed *in vitro* with virus and incubated 1 hr at room temperature before inoculation.

Therapeutic test = test material inj. 1 hr after infection.

Protection test = test material inj. 1 hr before infection.

infected with a sublethal dose (10 ID_{50}) of virus and were injected daily subcutaneously with one and 3 mg of APM, respectively, beginning one hour after infection. Twenty similarly infected but untreated mice served as the control group. All mice were sacrificed on the 10th day after infection and the degree of pulmonary consolidation was noted. The data indicate that daily injections of APM definitely suppressed the development of pulmonary lesions in mice infected with a sublethal dose of influenza A virus. However, it would appear that the effectiveness of APM in suppressing the development of pneumonia was not markedly increased when the size of the infecting dose of virus was greatly (1,000x) reduced (Table I).

It was of obvious importance to study the effect of APM on the rate of death of mice infected with a lethal dose of influenza A virus. The results of a typical experiment are summarized in Table III. This experiment was identical with the preceding one except that a lethal dose of virus (10,000 ID_{50}) was employed. The data show that daily injections of 3 mg of APM delayed death of the mice by approximately 2 days and that when the daily dose of APM injected was decreased to 1 mg the delay in death was reduced.

An attempt to demonstrate a reduction in the infective titer of pools of 8 lungs each collected 24 hours after infection was unsuccessful. The mice were injected subcutaneously with 5 mg of APM one hour before intranasal inoculation of 10 and 100 ID_{50} of influenza A virus, respectively.

Considerable time and effort were spent in attempting to demonstrate antiviral activity against influenza A virus in embryonated eggs using both culture filtrate and APM. Suppressive effects, though regularly observed, were minimal. The various preparations were rendered suitable for injection into eggs by the addition of penicillin (500 units per ml) and neomycin (200 units per ml). Control inocula were similarly treated. The results of several typical experiments are summarized in Table IV. The data indicate that culture filtrate known to be effective in suppressing the development of pulmonary lesions in mice was, at best, capable of effecting a slight (approximately 2-fold) reduction in the average hemagglutinin titer of allantoic fluid collected 48 hours after infection with 10-100 ID_{50} of influenza A virus. However, this slight reduction in the formation of viral hemagglutinin was obtained whether the culture filtrate was mixed *in vitro* with the virus or was injected one hour before or one hour after infection. It was hoped that APM would prove to be more effective than culture filtrate. However, this was not the case. A solution of APM

containing 5 mg per ml was sterilized with ethylene oxide(6), and was tested for antiviral activity by means of the contact test. As with culture filtrate a slight (approximately 2-fold) reduction in viral hemagglutinin was obtained. Attempts to demonstrate a reduction in the infective titer of allantoic fluid collected 24 hours after inoculation were unsuccessful. The eggs received potent culture filtrate one hour after infection with 10 ID_{50} of virus. It is clear that the embryonated egg was not a suitable host for the demonstration or detection of antiviral activity of APM against influenza A virus when the usual methods were employed.

APM and culture filtrate both failed to inhibit hemagglutination *in vitro* by influenza A or B viruses. Infected allantoic fluids were titrated in the usual manner(3) in the presence of culture filtrate, APM (2 mg per ml), broth and saline, respectively. The hemagglutinin-test material mixtures were incubated for one hour at 36°C before the addition of chicken erythrocytes. Identical end points were obtained in each of the various titrations with influenza A and B viruses, respectively.

Experiments with Newcastle disease virus (NDV). Ginsberg(7) has shown that the non-transmissible pneumonia in mice which follows intranasal inoculation of NDV paralleled the reactions between virus and host cell save that formation of new infectious particles did not occur. Further, when the maximum of pneumonia was observed there remained less than 0.1% of the original NDV inoculum and "immature or incomplete virus"(8-11) could not be demonstrated in lung tissues by complement fixation or hemagglutination. This unique host-virus relationship was found to be affected by APM in the experiments described below.

A large number of mice were inoculated intranasally with 0.1 ml of undiluted allantoic fluid containing 10^9 infectious units of NDV. Beginning one hour after inoculation and daily thereafter groups of 15 mice each were injected subcutaneously with various amounts of APM as indicated in Table V. Inoculated but otherwise untreated mice served as controls. All mice were sacrificed on the 3rd day after inoculation (at the time of maximum

TABLE V. Effect of APM on Development of Pneumonia Induced by Newcastle Disease Virus in Mice.

APM	Result—3rd day after inoc.* Lesion score I/T	L/M	%	Avg wt lungs, g
	0/4†			.18
Control	30/30	100/150	67	.27
1 mg SC × 3	12/15	20/75	27	.21
.3	13/15	20/75	27	.22
.1	14/15	19/75	25	.22
.03	10/15	18/75	24	.23
.01	12/15	34/75	45	.25

* 10^9 ID_{50}.
† Uninoculated controls.

pneumonia(7)) and the degree of pulmonary consolidation and the average weight of the lungs were recorded. It is clear from the data that daily injection of as little as 0.03 mg of APM suppressed but did not prevent the development of pneumonia.

No evidence of inactivation of NDV *in vitro* could be demonstrated when the virus was titrated in the presence of saline, culture filtrate, or APM, respectively, before inoculation into embryonated eggs.

Properties of APM. Preliminary qualitative data on the properties of APM were obtained in the following manner. Preparations of APM were treated as described below and tested for potency in mice previously inoculated intranasally with 10,000 ID_{50} of influenza A virus or 10^9 infectious units of NDV. One mg amounts of the various preparations were injected daily subcutaneously into groups of 12 mice each beginning one hour after instillation of virus. All mice were sacrificed on the 3rd day after infection and the degree of pulmonary consolidation and average weight of the lungs was determined (as exemplified in Tables I and V). The following results were obtained. The potency of aqueous solutions of APM was not completely destroyed by (a) exposure to a temperature of 120°C for 45 minutes at pH 9; (b) dialysis against running tap water for 24 hours; or (c) serial extraction of the same solution of APM with n-butanol, anesthetic ether, chloroform, petroleum ether, ethyl acetate and benzene. Half saturation of an aqueous solution of APM with ammonium sulfate resulted in the immediate formation of a flocculent precipitate which was

found to be both acid precipitable and potent.

No evidence of antibacterial or antifungal activity could be demonstrated when the streak-dilution method(12) was employed. A concentration of 5 mg of APM per ml of agar did not inhibit the growth of *Escherichia coli, Micrococcus pyogenes* var. *aureus, Pseudomonas aeruginosa, Shigella sonnei, Bacillus cereus, B. subtilis, Mycobacterium tuberculosis* (strain 607), *Streptomyces griseus, S. fradiae, Candida albicans, Aspergillus niger* or *Penicillium notatum.*§

Discussion. The most interesting characteristic of APM is its capacity to suppress the development of non-transmissible pneumonia in mice induced by the intranasal instillation of large numbers of infectious particles of NDV. Ginsberg(7) has suggested that the pneumonia following inoculation of NDV may be the result of the injurious action of a large amount of virus. However, he points out that the possibility of multiplication of non-infectious virus was not definitely eliminated. APM might affect either or both of these factors. On the other hand, it is equally plausible to visualize the action of APM as primarily affecting the lesion itself. It is clear that the *continued* presence of large numbers of infectious particles of NDV in the lung is not essential for the development of pneumonia(7). However, it is not known whether the development of the lesion is dependent on the continued presence of intracellular NDV in one form or another.

Attention should be drawn to the studies of Horsfall and Ginsberg with pneumonia virus of mice(13) and influenza A virus(5) as well as with NDV(7) which indicated that pulmonary lesions are formed at a rate different from that of infective virus. These investigators suggested that factors other than viral multiplication *per se* are involved in the production of lesions. The fact that APM suppresses viral pneumonia in mice in the presence or absence of the formation of new infectious virus is in agreement with this suggestion.

Studies on the mode of action of APM, its antiviral spectrum, and its chemical and physical properties are in progress.

Summary. A material (APM) produced by a culture of *Achromobacter sp.*, was found to possess a unique combination of biological characteristics. (a) APM was not inhibitory to representative species of bacteria or fungi *in vitro*. (b) Preparations capable of suppressing the development of pulmonary lesions in mice previously infected with influenza A virus were found to be devoid of virucidal activity *in vitro* and failed to affect the production of infectious virus in lung tissue or allantoic fluid. (c) The mouse was a far better host than the embryonated egg for the demonstration or detection of inhibitory effects of APM on influenza A virus. (d) Hemagglutination *in vitro* by influenza A or B virus was not affected by the presence of APM. (e) The most interesting characteristic of APM was its capacity to suppress the development of non-transmissible pneumonia in mice induced by intranasal inoculation of Newcastle disease virus.

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Received June 23, 1952. P.S.E.B.M., 1952, v80.

A Sensitive Gelatin Agglutination Test for Detecting Antisperm Agglutinins.* (19740)

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As a preliminary step in the study of antibodies against spermatozoa in fertile and sterile women it was necessary to develop a highly sensitive technic capable of demonstrating the presence of small amounts of antibodies. Among other serological tests the detection of anti-human sperm agglutinins offered a promising field, although previous investigators with mammalian spermatozoa had obtained only relatively low agglutination titers. Therefore, various methods of increasing the delicacy of the agglutination test by modifying the standard technics were tried.

These studies led to the development of a new type of test, the gelatin agglutination technic, which is capable of detecting sperm agglutinins in titers about 100-fold higher than with the ordinary agglutination tests. The technic is described since this test may have possible application in the detection of agglutinins against other organisms than spermatozoa. The rationale of the test is based on the use of live motile spermatozoa as antigens and the employment of a viscous medium in the form of 2.5% gelatin. This medium prevents the actively motile spermatozoa, once agglutinated, from disentangling themselves from the weakly bound floccules in high antiserum dilutions. It also retards sedimentation, thus rendering unnecessary redispersal of the delicately flocculated sediment.

Technic of test. Antigens. Donors with numerous motile spermatozoa and readily liquefying semen are used for the preparation of the antigen. Specimens of semen with large amounts of cellular debris are rejected to minimize nonspecific agglutination. A fresh ejaculum is allowed to stand until liquefaction is complete, usually in from 30 to 60 minutes. A sperm count is done and the specimen is diluted to approximately 60 million spermatozoa per ml with physiological sodium chloride solution or Baker's buffered glucose solution(1). The diluted semen is allowed to stand undisturbed for about an hour at room temperature to permit settling of clumped and immobile spermatozoa and cellular debris. The supernate, containing the actively motile spermatozoa, is carefully removed, a repeat sperm count is made, and the suspension is adjusted to 40 million spermatozoa per ml. An equal volume of gauze-filtered 10% gelatin in physiological sodium chloride solution is then added, giving a sperm concentration of 20 million per ml and a gelatin content of 5%. The gelatin solution and gelatin-sperm mixture are maintained at about 36-37°C to facilitate pipetting.

Test. Serial dilutions of the sera to be tested are made with physiological sodium chloride solution, the range depending upon the expected titers. A total of 0.3 ml of each dilution is first placed in a 65 x 5 mm precipitation tube and an equal volume of the spermatozoa-gelatin antigen is then added giving a final concentration of 10 million spermatozoa per ml in 2.5% gelatin. The use of these small precipitation tubes not only conserves reagents, but also provides a 30 mm column of fluid, which raises the sensitivity of the test by increasing the frequency of contact among the slowly sedimenting floccules. Comparison with the controls is also simplified, since the viscous gelatin medium tends to prevent settling of the unagglutinated spermatozoa. The contents of the tubes are well mixed and incubated at 36-37°C for 2 hours. This period is the most satisfactory from the standpoint of maximal agglutination and minimal sedimentation in the controls. The gelatin-prepared antigen plus diluent as well as known positive and negative sera comprise the controls. During incubation the antigen control tubes may be checked for the appearance of a granular precipitate, indicative of pseudoagglutination. Such nonspecific agglutination

* Under a grant from the National Committee on Maternal Health, Inc.

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occurs infrequently, but, if noted, incubation is terminated and the results of the test are immediately recorded.

Reading. At the end of the incubational period the tests are read. The tubes may then be placed, for subsequent observation, in the refrigerator, where rapid solidification preserves the agglutinates. The criteria for agglutination are the clarity of the dispersing phase and the size and character of the floccules. In true agglutination the dispersing phase is much clearer than in the controls. Finely dispersed granulations are occasionally encountered in negative serum controls but rarely in titers above 1:4, and are readily distinguishable from true agglutination. Final results are recorded as +, ±, and —, although positive reactions may be expressed in terms of 1- to 4-plus if desired.

Discussion. Antigen. The agglutination of human spermatozoa by antibody, while involving the same principles as the agglutination of bacteria, is modified by the number, the motility, and the anatomical characteristics of the spermatozoa. If too few spermatozoa are present in the antigen, the reduced frequency of collisons per unit volume results in the formation of small floccules. If too many are used, the amount of antibody available for each spermatozoon is reduced and an excessive turbidity is produced that may obscure small agglutinates in the higher serum dilutions. The optimal concentration is 20 million spermatozoa per ml for the antigen, which gives a final concentration of 10 million per ml in the test. There is no advantage in the use of washed spermatozoa over diluted whole semen for antigens, since the seminal plasma does not interfere with the reading of the test, when the final dilution of semen is 1:10 or higher. Actively motile spermatozoa are essential for the test. The higher the motility the greater is the number of collisons per unit time, thus facilitating agglutination. Furthermore, active spermatozoa give controls of homogeneous turbidity, whereas inactive spermatozoa show a greater tendency toward sedimentation. In the absence of the gelatin menstruum the large size of the spermatozoa causes agglutinates to sediment rapidly although the weakly bound floccules of intertwining heads and tails are easily disrupted by slight shaking and jarring, especially in the presence of small amounts of antibody where the sperm motility alone may be sufficient to overcome the agglutinative forces.

Gelatin menstruum. The viscous gelatin medium, while allowing the spermatozoa to agglutinate, retards their separation from the weakly bound floccules in the high antiserum dilutions and protects the agglutinates from mechanical shaking and jarring. The viscous medium also retards sedimentation in both the test and controls so that a direct comparison of the antiserum dilutions and controls may be made at the conclusion of incubation without resuspending the sediment. A gelatin concentration of 5% in the antigen (2.5% in the final dilution in the test) gives the most satisfactory results and is nontoxic to spermatozoa. Higher concentrations are impractical, since they are difficult to manipulate, and lower concentrations do not give sufficient viscosity to produce maximal titers. For example, when the same antisperm serum was tested against antigen suspensions which were prepared from one ejaculum but contained varying amounts of gelatin, the agglutinin titers were 1:8,000 for a 0.5% gelatin antigen, 1:32,000 for a 2.5%, and 1:128,000 for a 5%.

Application. The gelatin agglutination test, primarily designed for serological work with spermatozoal antibodies, has proved highly effective in detecting small amounts of antisperm agglutinins, since it gives titers about 100-fold higher than obtained with the usual agglutinative technics. The titers of 7 anti-human-sperm rabbits' sera with this technic ranged from 1:16,000 to 1:256,000 with a mean of about 1:60,000. The test appears to be highly specific since agglutination rarely occurs in dilutions above 1:4 with the normal sera of man, rabbits or guinea pigs, or with rabbits' antisera against various types of unrelated antigens. The gelatin test should prove of value in cases where the concentration of sperm agglutinins is too small to be detected by the ordinary macroscopical or microscopical agglutination tests. It should also be useful in the study of iso-agglutinins, and in the differentiation and determination

of relationship between the spermatozoa of different species.

The basic principle of the test, however, would seem to have a general application to any agglutinative technic involving large motile organisms, where the agglutinates formed are so fragile as to be readily disrupted by shaking or jarring. It may perhaps be of value in the study of trypanosomes and other large flagellated protozoa. Although gelatin has proved suitable for the enhancement of sperm agglutination by increasing the viscosity of the medium, different agents may be better suited for other motile organisms.

Summary. 1. An improved macroscopical agglutination test for the detection of agglutinins against human spermatozoa is described. 2. The test employs an antigen of actively motile spermatozoa, and a final concentration of 2.5% gelatin to increase the viscosity of the medium. 3. The viscous gelatin medium prevents dissolution of the weakly bound sperm agglutinates in the higher antiserum dilutions, and retards sedimentation so as to render resuspension unnecessary. 4. The new method appears to be highly sensitive and specific. Agglutinative titers ranging from 1:16,000 to 1:256,000 with a mean of about 1:60,000 have been obtained with 7 anti-human-sperm rabbits' sera. These titers are over 100-fold higher than those obtained with the usual agglutinative technics. 5. The possible applications of the test are suggested.

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Received July 22, 1952. P.S.E.B.M., 1952, v80.

Relation between Adrenal and Pituitary Glands and Urinary Excretion of Neutral Reducing Lipids in the Rat.* (19741)

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Adrenal cortical stimulation in the rat is, at present, evaluated by adrenal ascorbic acid or cholesterol depletion in acute experiments (1) and by adrenal weight and morphological changes in chronic experiments(2). During the course of studies on the role of the adrenal in experimental hypertension, it was thought desirable to evaluate adrenal cortical activity by measuring the urinary excretion of adrenal cortical hormones or their metabolites. Several chemical procedures(3-5) have been developed for the determination of these substances in human urine. The method of Heard and Sobel(3) is based on the ability of neutral lipid extracts of urine to reduce phosphomolybdic acid. This method has been shown to correlate with clinical and biological evidence of altered adrenal cortical activity in human beings. In the present study, a modification of this method has been applied to evaluate adrenal cortical activity in the rat.

Experimental procedure. Urine collection. The method for urine collection has been described(6). Rats of the Slonaker-Addis strain were taken off the stock diet and placed in urine collection cages at 5 P.M. During the overnight collection period the animals received *ad libitum* a 15% glucose solution in 0.4% sodium chloride containing 0.5% of a mixture of B vitamins (Betaplexin®). There may be some theoretical objections to the use of this solution in experiments designed to test adrenal cortical function. Nevertheless, it was necessary in order to assure a copious flow of uncontaminated urine. Normal rats on over-

* This investigation was supported by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service, and the L. B. Mayer Foundation.

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night collection drink approximately 50 to 60 ml of this mixture and excrete approximately 40 to 50 ml of sugar-free urine. Since the neutral reducing lipid material is rapidly destroyed in alkaline solution, 2 g of ammonium sulfate were added to the urine bottles prior to the collection period. The following morning the urines of each experimental group were pooled and filtered through glass wool. The pH of the pooled specimens was usually between 6.0 and 6.5. If the pH exceeded 7.0 the urine was discarded.

Chemical procedure. A 15 ml aliquot of urine was adjusted to pH 1.0 with 6 N hydrochloric acid with the aid of a Beckman pH meter. The urine was transferred to a 30 ml ground glass stoppered test tube and shaken with 4 ml of a 4:1 ether-chloroform mixture. The supernatant was aspirated into a separatory funnel. This operation was repeated 3 times. The combined extracts were shaken 4 times with 3 ml portions of ice cold 0.1 N sodium hydroxide and 4 times with 3 ml portions of water; then dried over anhydrous sodium sulfate. The extract was transferred to a colorimeter tube and the solvent was removed under a stream of nitrogen. Five ml of a 1:1 mixture of glacial acetic acid and phosphomolybdic acid solution (Folin-Wu) were added and the contents of the tube were heated for 60 minutes in a boiling water bath. A standard solution containing 100 γ of desoxycorticosterone was similarly treated. The color was read at 660 μ. The results were calculated as γ equivalents of desoxycorticosterone per rat per day. The calculated value is the amount of neutral reducing lipids (NRL) contained in the urine per rat per day.

Biological procedure. Bilateral adrenalectomy was performed in one stage under ether anesthesia. The adrenalectomized rats were maintained on a 1% sodium chloride solution for 4 to 5 days after the operation.

Beef pituitaries were carefully dissected and the anterior and posterior lobes were enucleated. The preparations were made by brief maceration of these fractions with acetone in a Waring Blendor. The residue was filtered, dried, then ground to a fine powder. These pituitary suspensions, as well as ACTH and growth hormone, were administered in 0.9% sodium chloride solution subcutaneously in the dosages indicated in the text. Control animals received 0.9% sodium chloride solution only.

TABLE I. Effect of Adrenalectomy and Administration of Pituitary Hormones on Neutral Reducing Lipid Excretion in the Rat.

Group	No. of rats	No. of observations	NRL (mean stand. dev.)		"p"*
Normals	600	150	61.2	2.1	
Adrenalectomy	104	26	29	1.9	.001
ACTH	74	14	74	8.1	.1
Growth hormone	27	7	91	13.9	.03
Ant. pituitary	24	6	78	6.8	.2
Post. "	24	7	191	17	.001

* "p" compares each mean to the mean of the normal controls.

Results. The data were analyzed by Fisher's Method. "p" values greater than 0.01 were not considered to be significant. The NRL excretion usually ranged between 50 and 90 γ per rat per day (mean 61.2 $\pm$ 2.1, Table I). However, since these limits were in some instances exceeded, it was necessary to compare the excretions of the experimental animals with that of their controls in any individual determination. There was no significant sex difference in the level of NRL excretion; male rats excreted 65 $\pm$ 2.7, while female rats excreted 57 $\pm$ 3.1 γ per rat per day. The difference between these means is not significant.

Adrenalectomy. The excretion of NRL by adrenalectomized rats was determined on 104 rats in 26 groups carried out over a period of nearly 2 years. The average daily excretion was approximately 29 γ per rat.

ACTH administration. It has been observed in this laboratory that rats of the Slonaker-Addis strain do not have an eosinopenia following a single injection of ACTH§ (7). It was necessary to give this substance in 3 doses in order to obtain a maximal eosinophile fall. ACTH was, therefore, given at 5 P.M., 11 P.M., and 7 A.M., during the overnight collection period. The total dosage

§ We wish to thank Doctors Edwin E. Hays and Irby Bunding of Armour and Company, Chicago, Illinois for supplying us with the ACTH and the growth hormone used in this experiment.

administered per rat ranged from 8 to 48 mg equivalents of LAIA standard. This was given to 74 rats in 14 groups. In only 3 instances was an average increase in NRL excretion obtained. It is concluded that ACTH does not significantly increase NRL excretion in the strain of rats used in this study.

Growth hormone administration. It has been suggested that growth hormone is involved in stimulating the production of mineralocorticoids by the adrenal cortex(8). Growth hormone§ was administered subcutaneously in doses of 0.5 mg to 12.5 mg per rat at the start of the urine collection period. In 6 out of 7 experiments, no significant change in excretion was noted. It is concluded from these data that growth hormone does not increase NRL excretion.

Administration of acetone dried anterior and posterior pituitary powder. Several pilot experiments indicated that saline suspensions of beef whole pituitary glands produced large increases in NRL excretion, whereas similarly treated suspensions of casein or rat liver powder in 0.9% sodium chloride solution were inert in this respect. Anterior and posterior pituitary gland preparations were then tested.

Fifty mg of preparation was suspended in 4 ml of 0.9% sodium chloride solution and injected subcutaneously at the beginning of the urine collection period. An equivalent amount of 0.9% sodium chloride solution was injected into the control rats.

Anterior pituitary powder produced no significant change in NRL excretion. Except in one instance, posterior pituitary powder produced excretions exceeding 160 γ per rat per day. The greatest quantity excreted by a group of normal rats was 124 γ per day. Following the administration of posterior pituitary preparation, the rats become lethargic for a period of several hours. This effect has been noted in dogs and rabbits and is described as a state of "apnea"(9). The output of urine was greatly decreased. In a single instance when NRL excretion did not increase following posterior pituitary injection, the oliguria and lethargy were still present. These symptoms were present after the administration of heated posterior pituitary suspension, although the preparation had lost, to a considerable degree, its ability to increase NRL excretion.

TABLE II. Effect of Posterior Pituitary Powder on Neutral Reducing Lipid Excretion in Adrenalectomized Rats.

Treatment*	No. of rats	Urine vol, ml/rat/17 hr	NRL, γ/rat/day
Normal	13	44	66
Normal post. pituitary powder	13	9	184
Adrenalectomy	18	34	36
Adrenalectomy post. pituitary powder	18	8	73

* All animals received 4 cc of .85% sodium chloride solution subcutaneously in addition to the indicated treatment.

Several preliminary experiments seem to indicate that pitocin‖ is inert, but that pitressin¶ does increase NRL excretion. Thus, totals of 7.5, 15, and 30 units of pitressin, given in 3 injections, caused an excretion of 95 γ, 144 γ, and 153 γ per rat per day. The control rats excreted 75 γ per rat per day.

Administration of posterior pituitary powder to adrenalectomized rats. It is possible that posterior pituitary powder increases NRL excretion by stimulating the adrenal cortex. In order to test this hypothesis, posterior pituitary powder was administered to adrenalectomized rats and the results are indicated in Table II. Posterior pituitary powder caused an average increase in excretion of 118 γ above normal while in adrenalectomized animals an average increase of 37 γ above the saline injected adrenalectomized controls was produced.

Discussion. The use of phosphomolybdic acid as a reagent for the determination of NRL is based upon the ability of the —CO CH_2OH group and the $\alpha:\beta$ unsaturated —3-ketone of ring A of the steroid nucleus to reduce the reagent(10). When applied to human urine, there is a correlation between the level of NRL excretion and the biological activity of equivalent extracts, although a fraction of the NRL is not of adrenal cortical

‖ Parke Davis brand used, 10 pressor units per 1 cc vial.

¶ Parke Davis brand used, 20 pressor units per 1 cc vial.

origin(3). The NRL determination in human urine has been used clinically to evaluate adrenal cortical function(11,12). The administration of ACTH to man causes a considerable increase in the excretion of neutral reducing lipids(13). Burstein(14) has reported that a large increase in excretion of NRL and formaldehydogenic material followed the administration of as little as 100 γ of ACTH to the guinea pig. It was, therefore, startling to observe that even though adrenalectomy reduces NRL excretion to half the control value, doses of ACTH which produce an eosinopenia in the Slonaker-Addis strain of rats do not increase NRL excretion. The failure of anterior pituitary powder to increase NRL excretion confirms the lack of effectiveness of ACTH.

It was even more surprising to observe that an increased NRL excretion was induced by posterior pituitary gland powder. The causative agent in this crude mixture appears to be thermolabile. The substance which increased NRL excretion probably acts through the adrenals, although some increase in excretion does occur in the absence of the adrenals.

Summary. The neutral reducing lipids were determined in the urine of rats. Adrenalectomy decreased the excretion of this material to one-half the control value. Neither ACTH, growth hormone, nor anterior pituitary powder significantly increased NRL excretion. Posterior pituitary powder increased the excretion approximately 3-fold. This response is considerably diminished in adrenalectomized rats.

We wish to thank the Glesby Brothers Grain and Milling Co., Monrovia, Calif. for supplying us with grain products.

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Received July 28, 1952. P.S.E.B.M., 1952, v80.

Influence of Influenza Hemagglutinin on Hemolysis Rates. (19742)

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Much of what is known about the red cell membrane has resulted from observing the effects of various chemical substances upon red cells. Certain of these when added to a cell suspension become attached to the cell surface. This attachment or its sequelae may manifest themselves in many ways, agglutination or lysis being among those most readily observed and least understood.

Although hemagglutinating and hemolysing substances are not always sharply differentiated, there are instances in which the two types of action can be separated quite clearly. In this report, which deals with the successive actions of the two types of substance, the guiding principle has been the idea that effects might be produced which could be interpreted as the result either of simple blockage of one function by the other or of complete independence of the two functions,

this being the simplest working hypothesis that occurred to us. It was realized, of course, that data might be obtained which would compel recognition of more complex interrelationships.

Methods. The choice of hemolytic and hemagglutinating systems was governed in part by considerations irrelevant to the foregoing argument. The PR8 influenza virus agglutinin was used because of an interest in the virus-cell interaction. It is not a typical hemagglutinin, because it produces irreversible effects upon the red cell surface which lead to its spontaneous elution. However, it can be converted by gentle heating into an agglutinin which attaches itself permanently to the red cell. Red cells treated with each of these substances were used in our experiments. The lysin, sodium choleate, was chosen partly for reasons of experimental convenience and partly because it is one of a class of agents that has been much investigated. Useful hypotheses are available concerning their mode of action. A *standard procedure* was used. Washed guinea pig red cells were mixed with the ultracentrifugally purified PR8 virus agglutinin or with the heated virus preparation to give a 1.5% cell suspension by volume, and from time to time the concentration of free agglutinin in the suspending medium and the rate of lysis of the red cells upon addition of sodium choleate were determined. The former was measured by titrating the agglutinin with chick red cells. The latter was determined turbidimetrically, the rate of lysis in the central portion of the sigmoid hemolysis curve being characterized by a unimolecular velocity constant k, the value of which is readily obtained by plotting the logarithm of the percentage of *intact* red cells against time and applying the following equation to the straight line so obtained:

$$2.3 \log (100\text{-}P) = 1n\ (100\text{-}P) = k\ (t\text{-}a)$$

P is percent hemolysis, t is time in minutes, and a is an intercept obtained by extrapolating the straight line to zero hemolysis.

Results. In nearly all experiments an unequivocal decrease in the constant k was observed during the adsorption phase of the adsorption-elution sequence. This was succeeded during elution by a gradual return to the normal value and then by a significant increase above the normal value. The magnitude of the effects observed can be seen from Table I in which the results of a representative experiment are given in condensed form. In this table the course of adsorption and elution of virus hemagglutinin can be followed by inspecting the column headed T/T_0 in which the amount of free agglutinin is expressed as a fraction of the total agglutinin present in the red cell suspension. The concomitant changes in lysis rate under standard conditions are shown in the final column, in which values less than unity represent inhibition of lysis and values greater than unity represent acceleration. It will be noticed that the process

TABLE I. Effect of Adsorption and Elution of Ultracentrifugally Purified PR-8 Influenza Virus Hemagglutinin on Unimolecular Velocity Constant for Lysis of Guinea Pig Red Cells by Sodium Choleate.

Time, min	T	T/T_0	k_0, min^{-1}	k, min^{-1}	k/k_0
−7	—	—	1.12	—	—
0	512	1	—	—	—
2	—	—	—	.67	.60
4	48	.09	—	—	—
16	—	—	—	.40	.36
17	48	.09	—	—	—
29	—	—	1.12	—	—
43	—	—	—	.62	.55
45	64	.13	—	—	—
54	—	—	1.12	—	—
126	—	—	—	.79	.75
138	—	—	1.05	—	—
178	—	—	—	.80	.78
181	384	.75	—	—	—
305	—	—	—	.70	.74
308	512	1	—	—	—
358	—	—	.92	—	—
363	—	—	—	.92	1
364	512	1	—	—	—
408	—	—	.83	—	—
413	—	—	—	.98	1.18
484	—	—	.79	—	—
491	—	—	—	1.18	1.50
494	512	1	—	—	—

Time scale is in min after addition of hemagglutinin to red cell suspension.

T is titer of free hemagglutinin in mixtures of red cells and agglutinin, determined with chick red cells by Salk method.

k_0 is unimolecular velocity constant for lysis of control suspension of guinea pig red cells by sodium choleate. Final concentrations in reaction mixture: red cells, .75 vol %, sodium choleate, .03%.

k is unimolecular velocity constant for lysis of guinea pig red cells in presence of PR-8 influenza virus hemagglutinin.

of elution and the corresponding change in lysis rate did not occur simultaneously. The first sign of reversal of the initial inhibitory effect was detected some time after elution had become evident, and the lysis rate constant continued to increase after complete elution.

The "nonelutable" heated hemagglutinin or "indicator virus" when used in the form of a solution of comparable titer to that of the original elutable material, although completely and permanently adsorbed by the standard guinea pig red cell suspension, produced only a slight and uncertain inhibition of lysis rate.

Discussion. In considering the interpretation of these results it must be recalled that whereas the lysis of red cells by substances like saponin and sodium choleate is without effect upon the average electrostatic charge density of the cell membrane(1), the adsorption and elution of influenza virus hemagglutinin is accompanied by a very great decrease in charge density(2). The discovery that lysis is inhibited when the agglutinin is adsorbed suggests that the sites of adsorption of lysin are situated somewhere on the virus receptor spots and may be the points of initial attachment of the virus agglutinin, this being followed by a chemical action involving a sufficiently large portion of the surrounding surface to cause a change in the average charge density of the entire surface.

This simple hypothesis of key spot blockade meets with some difficulty when the results obtained with the nonelutable agglutinin are considered. The proportion of the cell surface covered by the nonelutable substance must have been at least as great as the maximum proportion covered during the adsorption-elution of "active" virus, yet the inhibitory effect was slight. In future experiments attention will be given to two alternative hypotheses: (1) that the adsorption of heated agglutinin is less specific than that of the original substance; (2) that the inhibition in presence of the elutable material is caused by a reaction product and not by the agglutinin itself.

The results reported here are at first sight at variance with those of a single experiment described by Briody(3). However, his experiment differs, since he worked with a complex system containing uncontrolled amounts of several extraneous substances.

Summary. 1. The rates of lysis of guinea pig red cells by sodium choleate are altered by the adsorption and spontaneous detachment ("elution") of purified PR-8 influenza virus hemagglutinin. During the adsorption phase, lysis is inhibited; after detachment of the agglutinin, there is a delayed increase in rate of lysis to values above normal. 2. Considered in conjunction with electrophoretic data for red cells treated with virus hemagglutinin, these results suggest that the hemagglutinin first attaches itself to a lytic "key-spot". It then brings about, over a considerable neighboring area of the red cell surface, the changes which are manifested in alteration of the average electric charge density of the surface.

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Received June 9, 1952. P.S.E.B.M., 1952, v80.

Chemical Changes in Tumor-Bearing Mice.* (19743)

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It has previously been shown in this laboratory that significant changes occur in the nucleic acid content of the tissues of tumor-bearing mice(1-3). Similar changes, though less pronounced, were observed in mice during gestation(4). Evidence has also accumulated to show that the metabolic pattern of the organism as a whole is markedly altered by the presence of a malignant tumor(5,6). In view of the close relationship between the nucleic acids and protein synthesis during growth processes, it was considered of interest to study some of the amino acids in the tissues of sarcoma-bearing mice. A study of the individual purines and pyrimidines was also carried out, with a view of gaining some information regarding the composition of the tissue nucleic acids.

The data reported in the present paper show that along with the changes in the purine and pyrimidine bases, there occur alterations in the tissue proteins, as indicated by a significant increase in certain amino acids.

Experimental. Material and methods. Mice of the Swiss strain were employed in this study. Crocker sarcoma S-180† was transplanted subcutaneously into the right pectoral region of 5- to 6-week-old mice of mixed sexes. Groups of 40 animals were killed by decapitation at the end of 1, 2, and 3 weeks after implantation of the tumor. The separately pooled tissues were homogenized using distilled water at 0°C. (a) *Nucleic acids and purines.* The nucleic acids were extracted with hot trichloracetic acid(7). The pentose nucleic acid (RNA) was determined according to the method of von Euler and Hahn(8), and the desoxypentose nucleic acid (DNA) according to that of Stumpf(9). The remaining extracts were hydrolyzed with sulfuric acid (2), and guanine and adenine were determined according to the methods of Hitchings(10), and Woodhouse(11), respectively. (b) *Pyrimidines.* Estimation of pyrimidines was carried out as described previously(1), except that the chromatograms were developed in an iso-propanol-HCl medium(12). (c) *Amino acids.* An aliquot of each tissue homogenate, containing approximately 2 g of fresh tissue was adjusted with distilled water and concentrated HCl so that the final suspension had a volume of 25 ml and was 5 N in HCl. The tissues were hydrolyzed by autoclaving at 15 lb pressure for 8 hours. The hydrolysates were brought to a pH of 6.8 with NaOH solution and diluted to about 200 ml with a final concentration of approximately 2 mg of protein per ml. Arginine, histidine, and threonine were determined microbiologically with *Streptococcus faecalis*(13), while glycine was assayed with *Leuconostoc mesenteroides* P-60 (14). Cystine was determined colorimetrically by the method of Winterstein and Folin, as modified by Block and Bolling(15). The protein content was estimated by multiplying the Kjeldahl nitrogen value by 6.25.

Some difficulty was experienced in determining uracil in the lung, and thymine in the liver, since liver DNA and lung RNA are low. This difficulty was overcome by placing known amounts of standard solutions of uracil and thymine on the filter paper along with the hydrolysates. After developing the chromatograms the pyrimidine content of the tissues was determined by difference.

Results. In Tables I and II are presented the values for the nucleic acids, purines and pyrimidines in the sarcoma, and in the liver, lung, and kidney of sarcoma-bearing mice. The changes in the nucleic acids are reflected in the values for the purines and pyrimidines. Of significance are the changes in the pyrimidines, uracil, and thymine, which are a measure of RNA and DNA, respectively.

* This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research.

† Obtained from the Jackson Memorial Laboratory, Bar Harbor, Me.

TABLE I. Changes in Nucleic Acids and Purines in a Sarcoma and in Tissues of Sarcoma-Bearing Mice.*

Tissue	Wk after implantation of tumor	mg/g dry wt RNA	DNA	Adenine	Guanine
Sarcoma	1	52.5	43.5		
	2	44.7	38.3		
	3	31.2	36.2		
Liver	Control†	33.5 ± .9	9.8 ± .8	3.1 ± .1	6.4 ± .3
	1	40.2	12.3	5.8	8.9
	2	38.4	11.8	4.8	8.1
	3	40.2	17	5	7.5
Lung	Control	8.6 ±1.5	17.5 ± 2.2	3 ± .7	3.3 ± .6
	1	16.9	26.5	2.4	4.7
	2	14.7	33.9	3.8	5.9
	3	21.9	37	3.9	7.2
Kidney	Control	24.6 ± .9	21.9 ± 1.9	3.3 ± .1	5.6 ± .3
	1	21.5	21.5	3.9	5.9
	2	22.1	25.6	4.3	7
	3	24.3	33.7	5.3	7.7

* Values for RNA and DNA are taken from a previous publication (1).

† Values for controls are means ± stand. errors. S.E. $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$, where d is the deviation from the mean and n the number of determinations.

TABLE II. Pyrimidine Content of a Sarcoma and of Tissues of Sarcoma-Bearing Mice.*

Tissue	Wk after implantation	Uracil, μg/100 mg dry wt	% of RNA	Cytosine, μg/100 mg dry wt	Thymine, μg/100 mg dry wt	% of DNA
Sarcoma	1	166	3.2	671	480	11
	2	138	3.1	577	425	11.1
	3	125	4	477	427	11.8
Liver	Control	113	3.38	284	92	9.4
	1	168	4.18	391	116	9.4
	2	160	4.17	386	107	9.1
	3	169	4.18	422	158	9.3
Lung	Control	44	5.1	109	163	9.3
	1	93	5.5	191	237	8.9
	2	80	5.4	212	298	8.8
	3	99	4.5	264	333	9.1
Kidney	Control	129	5.2	228	215	9.8
	1	120	5.6	240	208	9.7
	2	134	6.1	244	218	8.5
	3	112	4.6	260	256	7.6

* All values are averages of triplicate determinations on a single hydrolysate.

In general, the increase in uracil follows the increase in RNA values, and that of thymine corresponds to the changes in DNA. It will also be noted that in the kidney the RNA content remains unchanged. The fact that the uracil concentration in this organ shows no significant change over the control is further evidence that only the DNA concentration increases in this tissue. When the values for uracil and thymine are expressed (Table II) as per cent of RNA and DNA, respectively, there is fairly good agreement among the thymine values for the liver, kidney, and lung, amounting to approximately 9% of the estimated DNA content. The corresponding values for the sarcoma are higher (about 11% of DNA). The values for uracil, however, show large variations.

Table III shows the percentages of the amino acids in the sarcoma and in the liver, lung, kidney, and spleen of sarcoma-bearing mice. In analyzing these data any change

TABLE III. Percentage of Amino Acids in Sarcoma S-180 and in Tissues of the Host.*

Tissue	Wk after implantation	Methionine	Arginine	Histidine	Threonine	Glycine	Cystine
Sarcoma S-180†	1	1.75 ± .05	4.56 ± .07	1.84 ± .02	3.40 ± .10	3.96 ± .06	1.11 ± .08
	2	2.15 ± .05	6.55 ± .09	2.06 ± .01	4.24 ± .09	4.76 ± .08	1.24 ± .11
	3	1.80 ± .07	6.11 ± .13	1.88 ± .03	3.99 ± .14	4.91 ± .10	1.19 ± .05
Liver	0	1.87 ± .05	4.15 ± .03	1.62 ± .01	3.47 ± .03	3.81 ± .05	.82 ± .05
	1	2.20 ± .03	5.30 ± .12	2.14 ± .01	3.74 ± .10	5.02 ± .04	1.18 ± .04
	2	2.01 ± .04	5.66 ± .04	2.02 ± .02	3.98 ± .15	5.66 ± .05	1.02 ± .07
	3	2.03 ± .04	6.06 ± .16	2.21 ± .04	4.43 ± .10	5.73 ± .01	1.23 ± .09
Lung	0	1.40 ± .03	3.43 ± .05	4.16 ± .06	3.95 ± .05	4.07 ± .06	1.06 ± .08
	1	1.50 ± .01	4.94 ± .17	3.90 ± .03	3.94 ± .07	4.90 ± .03	1.29 ± .08
	2	1.42 ± .02	5.41 ± .05	3.88 ± .03	4.30 ± .05	5.92 ± .10	1.35 ± .02
	3	1.57 ± .03	5.90 ± .09	3.46 ± .05	3.93 ± .04	5.32 ± .05	1.24 ± .01
Kidney	0	2.03 ± .05	4.68 ± .03	1.99 ± .03	4.70 ± .06	5.28 ± .14	1.26 ± .06
	1	2.12 ± .01	6.15 ± .17	1.92 ± .04	4.42 ± .06	4.90 ± .03	1.22 ± .04
	2	2.11 ± .04	5.60 ± .09	1.92 ± .03	4.50 ± .11	5.65 ± .10	1.27 ± .01
	3	2.11 ± .05	6.38 ± .13	2.08 ± .02	4.78 ± .08	5.31 ± .12	1.31 ± .02
Spleen	0	1.43 ± .02	4.46 ± .05	2.53 ± .06	3.52 ± .08	5.30 ± .05	
	1	1.39 ± .03	4.87 ± .04	2.28 ± .04	3.62 ± .04	—	
	2	1.57 ± .08	4.67 ± .07	2.30 ± .06	3.50 ± .11	5.31 ± .10	
	3	1.55 ± .02	4.69 ± .06	2.50 ± .05	3.97 ± .10	5.55 ± .14	

* Amino acid values expressed as % of total protein, the amount of protein having been calculated on the basis of 16% nitrogen.

† Values for each group are means ± stand. errors. S.E. $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$, where d is the deviation from the mean and n is the number of determinations. "n" = 8 for all amino acids except cystine, where "n" = 6.

less than 15% is not considered significant. In the sarcoma, the significant change in arginine and glycine may be noted. The values for the second and third week for these amino acids are higher than those for the first week. More interesting, and perhaps of greater significance, are the changes in the amino acids in other tissues. In the liver, the changes in methionine are not considered significant, but all the other amino acids show a definite increase over the normal controls. The maximum increases are: arginine, 46%; histidine, 36%; threonine, 28%; glycine, 50%; cystine, 50%. In the lung, an increase of 72% in arginine, 46% in threonine, and 27% in cystine was found. On the other hand, there is a progressive drop in the histidine content of the lung as the tumor develops. Arginine alone shows a significant increase in the kidney, whereas in the spleen none of the amino acids studied show a significant change.

Discussion. The changes observed in the pyrimidines and purines confirm our previous findings (1-3) that there is a significant increase in the nucleic acid content of the tissues of tumor-bearing mice. The increases in uracil and thymine parallel the observed changes in RNA and DNA, respectively.

It was hoped that the determination of the individual components of the nucleic acids would give some indication of the composition of the 2 nucleic acids in the various tissues. However, the values for uracil when expressed as per cent of RNA vary considerably in the different tissues. It must be emphasized that unless the 2 nucleic acids are separated, and the composition of each studied separately no rigid conclusions can be drawn. We plan to extend our studies along these lines. It is noteworthy that there is generally (except for the third week value for kidney, Table II) good agreement in the thymine values of the liver, lung, and kidney, when expressed as per cent of DNA. The values for the sarcoma are consistently higher. It will be noted that the nucleic acids were estimated colorimetrically by methods which are not highly sensitive. Secondly, the results are expressed on the basis of the dry weight of the tissue. Both of these methods leave much to be desired. In view of these limitations the agreement in the thymine values is remarkable. It is not

yet clear whether the composition of the nucleic acids in the sarcoma is different from that of other tissues. But in the case of the liver, lung, and kidney, it is concluded that the composition of DNA is the same. In this context the work of Chargaff and coworkers (16) should be mentioned. Their observations on isolated samples of DNA from a variety of sources suggest that the composition of this nucleic acid is constant for different organs of the same species and characteristic of the species.

The increases in amino acids might be ascribed to an increase either entirely in the free amino acids, in the protein alone, or in both. Preliminary studies on the liver in this laboratory‡ thus far indicate that the increase in the amino acids is not solely due to an increase in the free amino acids. Significant increases in the amino acids (including free amino acids) have been found to occur in neoplasms and in the tissues of newborn mice (17,18).

The fact that the amino acids in the spleen show no significant change may have a bearing on our previous observations(1-4) that the nucleic acid content of this organ does not show a definite increase as in the case of the other tissues.

Summary. 1. The nucleic acids, purines, pyrimidines, and methionine, arginine, histidine, threonine, glycine, and cystine have been determined in various tissues of mice of the Swiss strain bearing transplanted sarcoma 180 (Crocker). 2. The changes in the nucleic acid content of the tissues are paralleled by the values for the individual purines and pyrimidines. In the liver and lung of mice bearing sarcoma, there is an increase in both RNA and DNA, and this increase is further confirmed by the corresponding increases in uracil and thymine. The fact that only the DNA content of the kidney increases is further supported by an increase in the thymine values alone, while the uracil content of this organ remains unchanged. 3. The values for thymine when expressed as per cent of DNA, suggest that the composition of DNA in the liver, lung, and kidney is the same. 4. The study of the amino acids present in a sarcoma and in the tissues of normal and sarcoma-bearing mice reveals that there are significant changes in the amino acids of the tissues of tumor bearing animals. In the sarcoma, there is a significant increase in arginine and glycine, the values for the second and third week being higher than those for the first week. In the livers of these animals, there is a significant increase in arginine, histidine, threonine, glycine, and cystine over the normal controls. In the lung, the increase is noted in arginine, threonine, and cystine, while there is a drop in the histidine content, as the tumor progresses. Arginine alone shows a significant increase in the kidney, while in the spleen none of the amino acids studied shows any change over the normal control values.

‡ Experiments by Mr. E. J. Singer.

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Received June 11, 1952. P.S.E.B.M., 1952, v80.

Effect of Methionine upon Utilization of DL-Homocystine by *Brucella suis* (19744)

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Although homocyst(e)ine has not been isolated from biologic systems it is accorded an intermediate position in the present concept of the L-cysteine ⇄ L-methionine transformation(1). Available evidence indicates the participation of both these latter amino acids in protein synthesis. Hence, any organism capable of utilizing either as its sole source of sulfur is assumed to be capable of converting this compound to the other according to its needs. If such an assumption is correct, it seems reasonable that such an organism would be equally capable of utilizing an exogenous supply of the common intermediates, provided these encountered no permeability barrier in passage to the centers of cell metabolism. Conversely, the failure of a compound, designated as an intermediate, to serve as the sulfur source for this organism would cast suspicion upon its projected role in the transformation(2). It has been observed that certain Brucellae which utilize either L-cyst(e)ine or L-methionine alone, under conditions which do not permit utilization of DL-homocyst(e)ine, are "stimulated" to utilize the latter in the presence of trace amounts of methionine.

Experimental results. *Brucella suis* utilizes either L-cystine, L-cystathionine, or L-methionine as sole source of sulfur in a medium containing mineral salts, essential vitamins, L-asparagine, and lactate(3). Lactate plays an important, although undefined, role in the sulfur metabolism of these organisms, since its omission from the asparagine medium creates a requirement for cyst(e)ine. The asparagine and lactate components of the medium may, however, be replaced by an oxidized casein hydrolysate(3) without altering the capacity of *B. suis* to utilize L- or DL-methionine. Since growth was somewhat more rapid in the latter medium, it was substituted for the asparagine-lactate medium in certain phases of this study.

The experimental procedures and the sources of the *B. suis* cultures have been described previously(3). Each sulfur compound was added to the basal medium in a freshly-prepared, filter-sterilized solution. Its concentration in the medium is expressed in terms of micrograms or millimicrograms of sulfur per ml. The growth response of standardized inocula (100 to 200 thousand viable cells per ml) was determined turbidimetrically after 3

TABLE I. Relation of Methionine Contamination of DL-Homocyst(e)ine to Utilization by *Brucella suis*.

Homocyst(e)ine*		Estimated methionine content (% by) Microbiological assay†	Chromatography	Homocyst(e)ine required for growth of *B. suis* 1722‡ (μg sulfur/ml)
DL-Homocystine	Sample 1	5–10	5–10	2(±)
	" 2	5–10	5–10	2(±)
	" 3	<.1	0§	>10,<25
DL-Homocysteine		.25	±‖	>5
DL-Homocysteine thiolactone		.35		>5

* The DL-homocystine and the DL-homocysteine were commercial samples. The DL-homocysteine thiolactone was kindly supplied by Dr. R. P. Wagner, Department of Zoology, University of Texas.

† Based on assay with methionine-dependent strain *Brucella melitensis* 2460.

‡ When the homocyst(e)ine was supplied as the sole sulfur source.

§ No methionine spot detected.

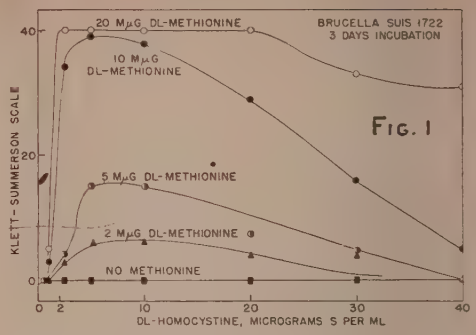
‖ Doubtful methionine spot.

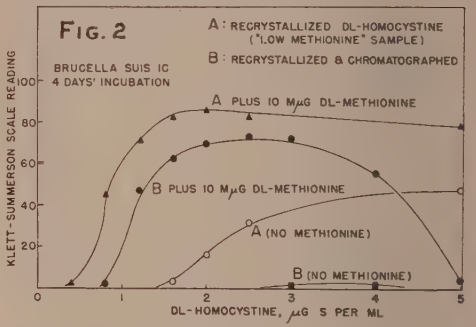
to 5 days incubation at 37°C.

Initial investigations of the availability of DL-homocystine* for 10 strains of *B. suis* yielded inconsistent results which were related to the different commercial samples under test. By paper chromatography, and by assay with a strain of *Brucella melitensis* which has an absolute requirement for methionine, a direct correlation was established between methionine content of the DL-homocystine samples and their utilization by *B. suis* 1722 (Table I). After recrystallization several times sample No. 3 yielded a product which, in the absence of added methionine, supported no growth of the test strain after 3 days' incubation with amounts up to 40 μg DL-homocystine sulfur per ml (Fig. 1). Other more sensitive strains, as *B. suis* 1C, required more rigid purification of homocystine in order to eliminate appreciable growth response in the absence of the methionine supplement (Fig. 2). Although DL-homocysteine and the thiolactone were not subjected to purification, their activity was no greater than DL-homocystine of comparable methionine content.

When 10 millimicrograms of DL-methionine per ml were added to the medium containing purified DL-homocystine, a striking growth response ensued (Fig. 1). This effect was detectable with as little as 1 millimicrogram of methionine sulfur, although for maximum response in the presence of 2 to 5 μg of DL-homocystine sulfur, 10 to 20 millimicrograms of methionine sulfur per ml were required. Although utilization of the impure preparations of DL-homocysteine and its thiolactone was stimulated by a supplement of 10 millimicrograms of methionine, utilization of lower concentrations was somewhat inferior to that obtained with DL-homocystine under comparable conditions.

The growth response to homocystine was roughly proportional to the concentration of methionine within the range from 1 to 10 millimicrograms (Fig. 3). It is significant that other known biological effects of amino acids can be detected only in quantities 10 to 100 times greater.





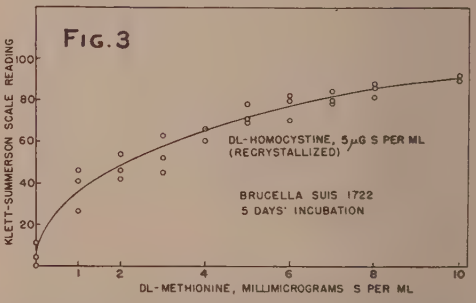


FIG. 1. Effect of methionine concentration on utilization and zonal toxicity of DL-homocystine. Tested in .1% oxidized casein hydrolysate medium.

FIG. 2. Effect of purification of DL-homocystine, and of added methionine, on growth in .1% oxidized casein hydrolysate medium.

FIG. 3. Relation of methionine concentration to utilization of DL-homocystine. Basal medium is .1% oxidized casein hydrolysate. 10 mμg DL-methionine alone produced no visible growth.

One of the unusual aspects of the growth response to DL-homocystine and DL-homocysteine in the presence of the methionine supplement was the form of the dose response curves. Relatively high concentrations of homocyst(e)ine were required before growth

* Commercial preparations, probably diasteroisomeric mixtures approximating 25% D-D, 25% L-L, and 50% D-L.

was initiated, and the subsequent response seldom was in direct proportion to increased concentrations of homocyst(e)ine. In some experiments it appeared as though there was a threshold concentration of homocystine below which it was not utilized, and above which assimilation was complete (Fig. 1). Although higher concentrations of methionine increased the sensitivity of response to homocystine, even an amount sufficient to produce detectable growth when used alone (about 20 millimicrograms per ml) failed to alter the sigmoidal character of the homocystine response curve. Moreover, at low concentrations of methionine the response curve indicates a zonal utilization of homocystine. If the concentration of homocystine exceeded that which produced the maximum response, a "toxic" effect became evident. This inhibition could be reversed by increasing the methionine concentration (Fig. 1). Riesen and colleagues (4) encountered a similar response of *Lactobacillus pentosus* to DL-homocystine.

D-, DL-, and L-methionine have been found equally effective in promoting utilization of homocystine in the asparagine-lactate medium. In the oxidized casein hydrolysate medium, however, D-methionine was inactive. By the addition of supplements of peroxide-treated casein hydrolysate to the asparagine-lactate medium it was demonstrated that the former inhibits utilization of D-methionine by *B. suis* 1722 and 1C. This inhibition can be reproduced with 100 μg of DL-methionine sulfone. From quantitative considerations, however, this sulfone cannot be considered as the only, or even the most potent, inhibitory substance in peroxide-treated casein hydrolysate. It appears likely that the oxidized casein inhibits conversion of D- to L-methionine, since *B. melitensis* 2460 utilized only DL- or L-methionine in its presence, or in the presence of DL-methionine sulfone. In the asparagine-lactate medium, however, the utilization of D-methionine by this organism is equal to or slightly superior to the utilization of L-methionine. Somewhat similar observations on the inhibitory effect of oxidized casein and methionine sulfone upon D-methionine assimilation have been reported for *Lactobacillus arabinosus* by Camien and Dunn(5).

The availability of DL-homocystine as a sulfur source in the asparagine medium was found to be dependent upon both the lactate and the trace methionine supplements. If either is omitted growth fails to occur. Oxidized casein hydrolysate, glutamic acid, and pyruvic acid substituted for lactate in promoting the utilization of homocystine to the same degree that they stimulated utilization of L-cystathionine and methionine. These results are consistent with the previous suggestion that lactate is linked specifically to a reaction transforming L-cystathionine to L-cysteine (3).

Another mechanism enabling *B. suis* to utilize DL-homocystine has been observed. Occasionally cultures incubated with recrystallized DL-homocystine produced delayed growth after 8 to 14 days. Transplants from such cultures utilize DL-homocystine promptly in the absence of methionine, although the growth shows the characteristic concentration-response lag of the "unadapted" culture (Fig. 4). This acquired capacity to utilize homocystine was retained during transfer in the absence of homocystine. It appears probable that a mutant capable of prompt utilization of homocystine was selected in the absence of methionine. On the other hand, transfers from cultures containing homocystine and as much as 8 millimicrograms methionine sulfur were no more efficient in utilizing methionine-free homocystine than was the parent culture. When the concentration of methionine in the original culture was reduced to 1 to 2 millimicrograms per ml the resulting growth contained a mixture of unadapted and mutant cells. Apparently the rate and quantity of growth in the original culture, as determined by its methionine content, is the principal factor operating for or against selection of the mutants capable of utilizing homocystine.

Consideration has been given to several possible explanations of the failure of purified DL-homocystine to function in the metabolism of *B. suis*. For example, inability to reduce the disulfide linkage, as observed with certain *Neurospora* and *Bacillus subtilis*(6,7) mutants, apparently cannot account for its inactivity for *B. suis*, since it is utilized no less efficiently than DL-homocysteine at compara-

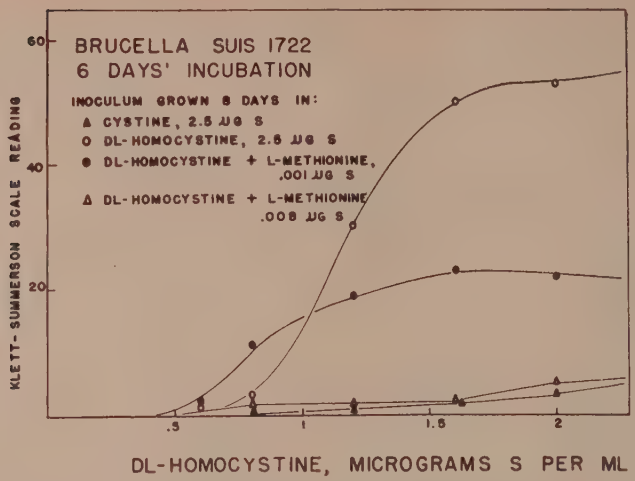


FIG. 4. Effect of inoculum source on utilization of DL-homocystine in a basal medium containing .1% L-asparagine and .01% lactic acid.

ble concentrations of methionine. Although membrane impermeability to pure homocystine cannot be ruled out, this explanation would require the difficult assumptions of an extreme degree of membrane selectivity for the next lower homologue, cystine, and the elimination of this selectivity by a minute amount of methionine. Moreover, it may be noted that homocystine utilization was not promoted by varying the pH of the medium over the range from 6.3 to 8.3, within which the response to cystine remain unaltered.

The possibility has been considered that the utilization of DL-homocystine is dependent upon an adaptive enzyme which requires a trace of preformed methionine for its synthesis. It is difficult, however, to reconcile this explanation with the prompt utilization of methionine-free L-cystine, which is assumed to pass through the homocysteine intermediate in its conversion to methionine. The same objection could be raised to the possible role of "active methionine"(8,9), functioning in a catalytic manner to convert homocyst(e)ine to methionine, thereby generating a continuous supply of methionine. It might be supposed that an "active homocyst(e)ine" is involved in the cysteine ⇄ methionine transformation effected by *B. suis*. Thus the mutant *B. suis* may possess an enzyme system for transforming DL-homocystine to the active form, whereas this mechanism is deficient or lacking in the wild type. This hypothesis has the merit of offering a possible explanation for the zonal toxicity of DL-homocystine in the presence of suboptimal concentrations of methionine, since it might be expected that an excess of DL-homocystine would compete with the "active intermediate" being formed slowly at low methionine concentration. However, it is also possible that the L-L form is the only isomer used in the diastereoisomeric complex, and that the other unnatural isomers (D-D and D-L) complete with the L-L form for essential enzyme systems. This inhibition might then be reversed by methionine, particularly within the "toxic" range of homocystine concentration.

Regardless of the explanation of the stimulatory effect of methionine upon utilization of homocystine by *B. suis*, it may be significant that other workers(10,11) have found that the amount of homocyst(e)ine required to produce maximum growth responses of different microorganisms exceeds by several fold the quantity of methionine or cystine required. It is possible that the excess was required to introduce sufficient methionine contamination to provide the stimulation necessary for efficient utilization of homocyst(e)ine. We have observed, for example, that the "methionineless" *Escherichia coli* mutant 58-161(11) grew

more promptly and at lower concentrations of "purified" DL-homocystine in the presence of 10 millimicrograms of methionine, although some delayed growth occurred in its absence. Certainly, conclusions relative to the metabolic utilization of homocystine by microorganisms must take into account the stimulatory effect of traces of methionine.

Summary. *Brucella suis* utilized either L-cystine, L-cystathionine, or L- or D-methionine as sole sulfur source in an asparagine-lactate medium. DL-homocystine, DL-homocysteine, and DL-homocysteine thiolactone also were utilized under the same conditions, but only to a degree related to their methionine content. Purified preparations of DL-homocystine were not utilized except in the presence of small quantities of supplementary methionine. D-, L-, and DL-methionine were equally effective in promoting utilization of DL-homocystine, and their effect increased to a maximum over a range from 1 to 20 millimicrograms of methionine sulfur per ml. Oxidized casein hydrolysate and methionine sulfone inhibited the activity of D-methionine. The mode of action of methionine and the possible significance of these observations are considered from the standpoint of the intermediacy of homocyst(e)ine in sulfur transformations.

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Received June 30, 1952. P.S.E.B.M., 1952, v80.

Immunization of Mice with Inactivated Type 2 Poliomyelitis Virus Against Types 2 and 3 Viruses. (19745)

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Homotypic immunization of mice with inactivated suspensions of Type 2 (Lansing) poliomyelitis virus has been reported(1-3). The amount of vaccine needed for definite protection of a mouse was derived from about 40 mg, or more, of infected central nervous system (CNS) tissue; Schwerdt *et al.*(4) however, immunized cotton rats with less material, about 10 mg (Loring *et al.*(5)).

Shortly after the MEF1 strain, Type 2, of poliomyelitis virus had been successfully adapted to newborn mice(6,7), and after it was found that sera from persons having Type 1 (Brunhilde) virus infection but no past or concurrent infection with Type 2 virus reacted with a complement-fixing antigen prepared with the adapted MEF1 strain(8), the present studies were begun. They concerned the possibility of an increased immunizing power developed by the newborn-mouse adapted MEF1 virus and of cross-protection after vaccination with it against types other than Type 2. In the studies reported here, therefore, vaccines were prepared using both the newborn-mouse adapted MEF1 strain and the standard, unadapted, virus. The MEF1 standard (Type 2) and the Leon (Type 3) were used as challenge agents. The reason for selecting Type 3 as the heterotypic agent was that of expediency, namely, the Leon strain can be propagated in mice by the intraspinal

* Dr. Brown's work was aided by a Fellowship from The National Foundation for Infantile Paralysis.

TABLE I. Vaccination of Mice with Inactivated Suspension of MEF1 Strains of Poliomyelitis Virus. Challenge with Standard MEF1 Strain.

Exp. No.	Vaccine, 10% brain suspension: Strain	LD_{50}	Inact.	Test for virus	Amt inj. per mouse, mg of tissue	Challenge	Result: Log −1	−2	−3	−4	−5	LD_{50} titer log	LD_{50} control minus test. log	Protection index‖
1	Adapt.	3.2	F*	0/10§	60	I.cer	0	0	0	0		<.5	>3.2	>1600
	Stand.	3.2	F	0/12	60		3/9	0	1	2		1	2.7	500
					0		10	8	8	5	1	3.7		
2	Adapt.	——	Same as Exp. 1	——	10	"	1/9	1	0	1		<.7	>1.9	>80
	Stand.				10		5/9	6	4	1/9		2.3	.3	2
					0		4/7	9	3	2	0	2.6		
3	Adapt.	—	F	0/10	10	"	3/8	5	0	0		1.4	1.9	80
	Stand.	—	F	0/10	10		8	7	4	4/9		2.8	.5	3
					0		10	8	6	3	1	3.3		
4			F	0/12	10	"	0	0	0	0		<.5	>2.9	>800
					1		4	2	0	1		1.1	2.3	200
	Adapt.	5.6	F†	0/12	10		2/9	2	0	0		<.8	>2.6	>400
					1		3	4	3	0		1.4	2	100
					0		6/9	8	8	4	1	3.4		
5	Adapt.	5.4	UVL‡	0/18	10	"	2	1/8	0	0		.7	3.3	>2000
					1		4	6	5	0		2.1	1.9	80
					.1		10	10	10	8		4.4	−.4	<1
					0		9	10	9	5	2	4		
6	Adapt.	———	Same as Exp. 4	———	10	I.spinal	7/12	5/13	1/12	0/13		1.6	2.4	250
					0		9/9	9/10	6/9	5/9	3/10	4		

* F = Formalin, .4%. † F = Formalin, .4%, incubated for 17 hr at 37°C. ‡ UVL = Ultra-violet light irradiated. § No. of animals paralyzed or dead over No. inoculated. Under result, 10 mice were inoculated for each dilution unless otherwise stated; *e.g.* 1 = 1 mouse died of 10 inoculated; 1/9 = 1 died of 9 inoculated. ‖ Protection index = antilog of the difference between log LD_{50} of control minus log LD_{50} of test animals.

route of inoculation (9). Studies on Type 1 virus used for challenge are also under consideration.

The properties and general manner of handling the newborn-mouse adapted variant of MEF1 virus have been described (6,7).

Vaccines. Suspensions used for vaccination were prepared as a 10% suspension of infected brain tissue (at first cord was also used) in physiological saline solution. A Waring Blendor homogenized the suspension and inactivation was carried out usually by addition of commercial formalin in final concentrations of 0.2 to 0.4%, and in a few instances by irradiation with ultraviolet light.† The formalinized vaccines were held at 4°C (in one instance after incubation at 37°C for 17 hours) (Table I) and were tested for activity usually at weekly intervals by intracerebral inoculation‡ into 8-15 mice. The mice were held under observation for at least 28 days. Failure of any mice to show neurological signs or to succumb was taken as an indication of inactivation. It is to be noted that this time (28 days) of observation was therefore added to the time required for inactivation by formalin before the vaccine was used in preventive inoculations. As will be seen later, on 2 occasions only, Exp. 7 and 8 (Table II), traces of active virus were found. In Tables I and II are shown the pertinent data for each vaccine, including the LD_{50} titer of the virus before inactivation, concentration of formalin used, time elapsed between the preparation of the vaccine and its preventive inoculation, and the number of mice that showed CNS signs or died following the intracerebral injection of the undiluted (10% CNS tissue) vaccine in the test for the presence in it of active virus.

Immunization. Mice were chosen when 30 to 40 days old and collected in 2 or more groups: control, and vaccination. The vaccine, adequately diluted, was given intraperitoneally in 0.1 or 0.2 ml twice or thrice at 7-day intervals. The animals were challenged 8 to 10 days after the last injection. The total amount of vaccine given to each mouse is

† We are grateful to Dr. George I. Lavin of the Rockefeller Institute for his generous cooperation.

‡ All such experimental procedures were performed with the aid of ether anesthesia.

TABLE II. Vaccination of Mice with Type 2 Newborn-Mouse Adapted MEF1 Strain and Intraspinal Challenge with Type 3 (Leon) Virus.

Exp. No.	Vaccine, 10% brain suspension: Passage No.	LD_{50} titer log	Inactivation	Days after prep.	Test for virus	Amt inj per mouse, mg of tissue	Result: No. mice	% dead or paral. at day 7	14	21	28	35	At end of exp.: No. dead	No. paral.	No reaction	Mortality: χ^2	P
7	110	5.7	F. .2%	8	3/7	60	71	39	39	30	27	25	1	17	53	44.6	p = <.001
						0	77	77	88	90	88	88	40	28	9		
8	113	5.9	F. .2%	33	7/28	60	59	42	44	29	24	25	1	14	44	6.2	.05>p>.01
						10	63	40	30	22	21	18	0	11	52	9.2	.01>p>.001
						0	60	67	75	73	70	65	10	29	21		
9*	76	5.6	F. .4%	180	0/12	20	23	30	35	30	26	26	0	6	17	6.3	.05>p>.01
	89	5.4	U.V.I.	60	0/18	20	30	33	43	33	20	20	2	4	24	4	p = .05 app.
						0	26	46	65	65	65	65	8	9	9		
10	114	5.5	F. .3%	66	0/30	10	30	53	57	57	57	57	0	11	19	12.1	p = <.001
						0	34	73	85	85	85	85	13	16	5		
11	115	5.6	F. .4%	71	0/14	20	33	43	33	33	27	27	0	9	24	11.5	p = <.001
						0	28	71	79	75	71	75	10	11	7		
12	123	5.6	F. .3%	43	0/15	10	25	8	8	8	8	8	0	2	23	5.6	.05>p>.01
						0	26	38	42	54	54	54	7	7	12		

* Exp. 9 35-42 days under observation; abbreviations as in Table I.

shown in the tables as the number of mg of infected tissue contained in the total inoculum, 1 ml of 10% vaccine containing 100 mg.

Virus used for challenge and route of inoculation. The MEF1 standard strain was given by the intracerebral route in the usual manner. The Leon strain adapted to mice by Habel and Li(9) was inoculated intraspinally as a 10 or 20% suspension of infected cord tissue. This strain in its 50th passage in mice was sent to us by Drs. Habel and Li, to whom we express our thanks. It has undergone 6 additional passages in this laboratory. In our hands the strain showed the properties described by Habel and Li(9): it was pathogenic only after intraspinal inoculation; virus could be recovered from the spinal cord but not from the brain in affected animals; at dilutions of cord greater than 10^{-1}, mice were rarely affected; the incubation period was 3 days or more, usually from 4 to 10; finally, following inoculation of 10 or 20% suspension, the morbidity in the present tests was 50 to 90% and mortality, 15 to 55%. When the Lansing virus was injected intraspinally into mice, its properties differed wholly from those of the Habel-Li Leon strain(9): the incubation period was shorter, 2-4 days; death almost invariably followed paralysis; and the LD_{50} titer was $10^{-4.0}$ or $10^{-4.5}$. It is clear, therefore, that there was no admixture of viruses in the Leon strain here used and that it retained its characters as defined by Habel and Li.

Results. Table I is illustrative of experiments in which mice vaccinated with the newborn-mouse adapted or with the standard MEF1 virus were challenged with MEF1 standard strain by the intracerebral route, except in one instance of challenge by the intraspinal route. It is plain that a vaccine prepared with the standard MEF1 strain protected mice when the total amount of vaccine given each mouse was equivalent to 60 mg of infected tissue, but no protection was elicited when 10 mg of tissue were given. In contrast, when vaccines were prepared with the newborn-mouse adapted variant of MEF1 strain, as little as 1 mg of infected brain tissue, equivalent to 0.01 ml of a 10% suspension, gave protection against 80 to 200 LD_{50} of virus; 0.1 mg of tissue, on the other hand, failed. Protection could also be achieved against challenge inoculation given by intraspinal route, as shown in Exp. 6 (Table I).

Table II is a summary of tests in which vaccines derived from the MEF1 variant virus only were used, and the challenge was carried out by intraspinal inoculation of mouse-adapted Leon virus. As indicated above, the low degree of pathogenicity of the Leon strain for mice prevented inoculation of serial dilutions of virus; hence the challenge in the experiments reported in Table II comprised 10 or 20% dilutions of virus in a volume of 0.02 ml. Following this intraspinal inoculation, approximately 10% of the mice died immediately, and an additional 10 to 20% of the animals showed paralysis of one or more limbs or marked scoliosis immediately after inoculation, which at times persisted for several days, or even indefinitely. In order to distinguish this type of paralysis induced by technic from that following virus activity, all mice that were paralyzed 24 hours following intraspinal challenge were discarded. Specific paralysis first appeared on the 4th day, rarely on the 3rd, as already indicated. The paralysis in the control animals was of a progressive type, beginning usually in one limb and extending to all extremities within the next 5 or 6 days. The paralysis was flaccid, and was accompanied by considerable atrophy of tissue and loss of weight. Control animals that survived after being paralyzed rarely recovered mobility of the affected limbs. In contrast, vaccinated mice that became paralyzed showed a limited extent of paralysis involving usually only one or 2 limbs, rarely more, and within 8 to 10 days developed recognizable recovery of function. With time more and more animals recovered completely, often as many as half the number paralyzed within the first 7 days. Little or no loss of body weight was noted.

Exp. 7 and 8 (Table II) were carried out with formalinized suspensions which were not ripened long enough for the virus to become completely inactivated. Thus, in Exp. 7 the formalinized vaccine, undiluted and also in dilutions of 10^{-1} and 10^{-2}, was tested for viral activity. Three of 7 mice died when given

undiluted vaccine intracerebrally but none in the groups of 10 mice receiving diluted vaccine. It is of interest that 8 days after preparation of this vaccine, the first dose contained less than one infective unit of virus; when the second and third injections were given 7 and 14 days later, no active virus was present in the undiluted vaccine. Similarly, the vaccine used in Exp. 8, tested undiluted 33 days after preparation, killed 7 of 28 mice. None of all the other vaccines had any recoverable virus at the time when used for the immunization test. There was no significant difference in results, therefore, when a minute amount of active virus was by chance present in the vaccine.

The type of immunity induced by formalinized vaccine is well exemplified by the data in one of the typical experiments, Exp. 7 (Table II). The control animals, once paralyzed, either died or remained paralyzed during the period of observation. In contrast, in vaccinated mice morbidity reached a peak of 50% at 8 days, then diminished gradually, so that at 35 days only 25% of the mice were still paralyzed, the others having recovered. In the same experiment, furthermore, the control animals showed an average loss of 13% of their body weight by the 21st day after challenge, with no subsequent gain or loss. The vaccinated mice, on the other hand, showed no loss of weight by the 21st day and an average gain of 5% by the 35th day. The χ-square test, with Yates correction, applied to the figures of mortality in control and vaccinated animals, exhibited a significant difference in all experiments (Table II).

As the quantity of inactivated vaccine given was small, it became of interest to find out whether circulating neutralizing antibody was produced. To this end, 6 mice from each of the vaccinated and control groups (Exp. 11) were bled under ether anesthesia, on the day of challenge. The sera were tested for neutralizing antibody against the standard MEF1 virus both by means of virus and of serum dilutions. It was found that the undiluted serum from vaccinated mice had a neutralization index of 160, or greater, and that the titer of this serum was $10^{-0.8}$, when tested against 40 LD_{50} virus. In tests for neutralizing antibody against the mouse-adapted Leon strain, no satisfactory results could be secured because of the failure of controls to react regularly even with low dilutions of the virus. A test using the monkey or tissue culture is therefore indicated.

Discussion. It is apparent from the data reported here that the immunogenic potency of the newborn-mouse adapted MEF1 strain of poliomyelitis virus is far greater than that of the standard, nonadapted parent strain, when tested under similar conditions. Thus it was found that an amount of inactivated vaccine deriving from 60 mg of CNS tissue infected with the standard MEF1 strain was needed to induce significant protection; on repeated occasions, 10 mg of this strain failed to protect even against 3 or 4 LD_{50} of virus. In contrast, as little as 1 mg of tissue infected with the adapted strain was sufficient to give a significant, reproducible protection against a homotypic challenge with Type 2 (MEF1) strain of poliomyelitis virus.

The low degree of pathogenicity of the Leon strain (Type 3) for mice precluded quantitative studies. It was evident, however, that there was a certain degree of cross-protection against this Type 3 virus when mice were vaccinated with relatively small amounts of inactivated MEF1 newborn-mouse adapted virus. Thus, of a total of 141 mice vaccinated with 10 or 20 mg of completely inactivated virus (Exp. 9, 10, 11, 12, Table II) only 2 died following intraspinal inoculation of the Leon strain; while in the same experiments 38 of 114 unvaccinated controls died after the challenge (χ-square $= 46.1$; $p < 0.001$).

The fact that large numbers of vaccinated mice developed only transient paralysis when challenged intraspinally with the Leon strain might be interpreted to indicate that there was a degree of virus multiplication which, however, was inhibited at a certain point with result that the extent of paralysis was much less and its duration shorter than in control mice. In controls, on the contrary, the infection was infrequently self-limited and often progressed to cause death. It is not possible at the moment to declare whether this type of resistance in the vaccinated is based on tissue immunity or on circulating neutralizing an-

tibody. Homotypic neutralizing antibody was found in the serum deriving from vaccinated mice; as for heterotypic antibody, experimental limitations prevented a clear-cut result indicating the significance of neutralizing antibody against Type 3 virus. It is of interest to recall in this connection that Sabin (10) found cross-neutralizing antibody to arise in man against Type 2 virus during the early stage after infection with Type 1 poliomyelitis virus. Whether the cross-protection induced with the inactivated MEF1 newborn-mouse variant would extend to include Type 1 has not as yet been investigated. Studies similar to the ones reported here would be indicated in monkeys with Type 1 virus; in these animals the oral route of infection rather than the intracerebral may possibly be used quantitatively(11). In this case more proper conditions for detecting any cross-protection between Types 1 and 2 might be available since it is known that with several other neurotropic viruses a low or even negligible degree of resistance against an intracerebral challenge proves to be considerable on challenge by peripheral routes.

Finally, in view of the greater degree of multiplication of the newborn-mouse adapted MEF1 virus in monkey testicular tissue culture(12), it is suggested that use of the adapted strain in such cultures might prove advantageous for studies similar to those reported here.

Conclusions. Experiments were carried out in mice in which an inactivated suspension of the MEF1 newborn-mouse adapted strain of Type 2 poliomyelitis virus was used as an immunizing agent. It was found that 1) the adapted strain had a far greater immunogenic potency than the standard when the animals were challenged with the homotypic strain; and 2) a significant and reproducible cross-protection was detected when the mice were challenged intraspinally with a heterotypic, Type 3 (Leon) strain.

The authors are grateful to J. J. Gingell and H. Koch for their valuable technical assistance.

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Received July 2, 1952. P.S.E.B.M., 1952, v80.

Statistical Analysis of Renal Clearance by the Dog.* (19746)

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A lack of adequate references in the literature to the values of normal renal function in the dog made it apparent that a statistical analysis on a large number of data obtained in this laboratory might be of general use. Therefore, a statistical analysis of "normal" renal clearance was performed on the data accumulated over a period of 6 years using 31 female dogs. The measurements of renal function included glomerular filtration (creatinine clearance)(1), p-aminohippurate (PAH) renal plasma flow and Tm(2), arginine Tm(3), and "filtration fraction" of penicillin-G clearance(4,5) compared with filtration fraction of para-aminohippurate clearance. No attempt was made to replicate

TABLE I. Mean Creatinine Clearance Values for Dogs, Expressed as ml/min/M².

Dog	Year 1943 mean	1944 mean	1945 mean	1946 mean	1947 mean	1948 mean	1949 mean	Grand mean
1	80 (51)*	83 (75)	76 (76)	91 (44)	100 (21)	—	—	83
2	121 (2)	—	—	—	—	—	—	121
3	89 (2)	—	—	—	—	—	—	89
4	67 (5)	—	—	—	—	—	—	67
5	—	83 (45)	115 (11)	—	—	72 (2)	—	88
6	82 (59)	86 (72)	105 (15)	97 (94)	101 (77)	96 (40)	74 (25)	92
7	—	84 (57)	59 (8)	—	—	—	—	81
8	—	64 (2)	102 (14)	—	—	—	—	97
9	88 (13)	—	—	—	—	—	—	88
10	—	125 (8)	—	—	—	—	—	125
11	—	—	113 (16)	—	—	—	—	113
12	—	—	100 (18)	—	—	—	—	100
13	—	—	80 (34)	94 (38)	93 (31)	75 (6)	—	88
14	71 (2)	—	91 (38)	86 (73)	86 (23)	—	—	87
15	—	—	113 (4)	—	—	—	—	113
16	—	—	89 (3)	—	—	—	—	89
17	—	—	83 (3)	107 (83)	114 (80)	—	—	110
18	—	—	87 (3)	94 (78)	92 (79)	79 (24)	—	91
19	—	—	—	—	101 (6)	59 (2)	—	90
20	—	—	—	—	170 (3)	—	—	170
21	—	—	—	—	—	98 (3)	—	98
22	—	—	—	111 (13)	113 (18)	70 (9)	—	103
23	—	—	—	—	124 (8)	—	—	124
24	—	—	—	—	—	93 (77)	92 (21)	93
25	—	—	—	—	120 (2)	116 (3)	—	118
26	—	—	—	90 (16)	91 (47)	87 (18)	81 (4)	90
27	—	—	—	99 (18)	104 (60)	142 (12)	—	108
28	—	—	—	—	110 (12)	109 (77)	—	109
29	—	—	—	—	—	104(131)	90 (14)	102
30	—	82 (10)	—	—	—	—	—	82
31	—	—	—	—	—	—	74 (12)	74
Grand mean	82	85	89	96	101	99	83	$\bar{\bar{X}} = 94$

* No. of determinations.

the experiments equally for all dogs, since some of the animals were not always available for experimentation over the entire range which is covered by this study. All values presented here are as per sq. M of surface area, calculated from the Meeh-Rubner equation:

$$SA = \left(\frac{11.2 \times W^{2/3}}{100} \right)$$ where SA = surface area, and W = wt in kg

The determination of all creatinine for measurements of glomerular filtration in this study was obtained by the method of Folin and Wu(6). Para-aminohippurate values were obtained by the method of Smith *et al.*(2). All arginine values were determined by the method of Stokes *et al.*(7). In the course of these studies the penicillin assays were those of Rammelkamp(8), Schmidt and Moyer(9), and Oliver(10).

A test for interaction between dogs and years was determined on a total of 2,045 determinations of creatinine clearances using a weighted method described by Snedecor(11). It was found by this analysis that large discrepancies existed among dogs from year to year (Table I). The standard deviation of a single determination on any given dog for any one occasion was 9 cc/min./sq. M surface area. The 95% confidence limits were 18 cc/min./sq. M surface area. Ninety-five per cent of all observations in this study may be expected to fall within the limits 94 ± 36 cc/min./sq. M surface area.

The analysis of 290 determinations of p-aminohippurate renal plasma flow showed that the mean values differed from dog to dog and from one occasion to another (Table II). The standard deviation of a single determination

TABLE II. Mean p-aminohippurate Renal Plasma Flow Values for Dogs, Expressed as ml/min/M².

Dog No.	1	2	3	4	5	6	7	8	9	10	11	12	$\bar{\bar{X}}$
Mean value	160	184	244	283	211	279	226	324	199	285	233	283	238
No. of determinations	20	47	103	2	3	6	3	17	9	2	32	46	290

TABLE III. Analysis of Variance.

Source of variation	Degrees of freedom	Sums of squares	Mean square	F ratio*
Between dogs	6	54.9043	9.1507	2.83
Within dogs	13	41.9710	3.2285	
Total	19	96.8753		

* F ratio = Ratio of mean² for between dogs to the mean² within dogs.

on any given dog for any one occasion was 32 cc/min./sq. M surface area. The 95% confidence limits were 63 cc/min./sq. M surface area. Ninety-five per cent of all observations in this study may be expected to fall within the limits 238 ± 133 cc/min./sq. M surface area.

It was not possible to do an extensive analysis of the p-aminohippurate Tm values, since only 20 values spread over 7 dogs were available. In this analysis the variation between dogs was not significant and could be attributed to experimental error (Table III). The standard deviation of a single determination on any given dog for any one occasion was 1.8 mg/min./sq. M surface area. The 95% confidence limits were 3.9 mg/min./sq. M surface area. Ninety-five per cent of all observations in this study may be expected to fall within the limits 16.5 ± 4.7 mg/min./sq. M surface area.

The analysis of arginine Tm was made on 27 values from dogs variously employed over the years 1946, 1947 and 1949. The statistical method used was an adaptation of a procedure of Ganguli(12). The mean arginine Tm value for 1949 was significantly lower than that for 1946 and 1947. The difference between dogs was not significant and may be a reflection of differences between years. Since the difference between years was significant, the estimation of a general mean arginine Tm value is not too meaningful. We report it here for completeness (Table IV). The standard deviation of a single determination for any given dog for any one occasion was 1.7 mg/min./sq. M surface area. The 95% confidence limits were 3.3 mg/min./sq. M surface area. Ninety-five per cent of all observations in this study may be expected to fall within the limits 12.9 ± 7.6 mg/min./sq. M surface area.

There was a total of 919 values on 10 dogs available for study of the "filtration fractions" of penicillin clearances as compared to the filtration fractions of p-aminohippurate clearances. It was evident from a preliminary analysis on all of the values that the variations among the filtration fractions of penicillin and the filtration fractions of p-aminohippurate values from dog to dog and from one occasion to another were greater than those observed in the same dog on the same occasion. Therefore, replicate determinations for every occasion were averaged and these values (63 for filtration fraction of p-aminohippurate and 207 for filtration fraction of penicillin) were used in the actual analyses. Because of the great disproportionality of the data, a weighted analysis of variance was done according to the method of Snedecor(11) (Table V). The weighted mean value for the filtration fraction of p-aminohippurate was .40. The difference between the weighted value of penicillin and the weighted value of p-aminohippurate was .05. In general, there is a consistent trend for the filtration fraction of p-aminohippurate values to be slightly higher than the filtration fraction of penicillin values. This is highly significant statistically ($P < 0.01$ and close to $P = 0.001$). Actually, in spite of the significant statistical result, we would require more data in the filtration fraction of the p-aminohippurate group of figures to be sure that differences noted are real and not a reflection of disproportion.

TABLE IV. Mean Arginine Tm Values for Dogs, Expressed as mg/min/M².

Dog No.	Date of exp.	Arginine clearance values: Mean value for date	Mean value for year	Mean value for dog
370	9–17–46	15.26	15.55	17.27
	9–19–46	15.84		
	10–30–47	20.71	20.71	
365	9–17–46	13	12.07	12.23
	9–19–46	11.14		
	10– 2–47	16.08	14.27	
	11–13–47	13.07		
	6–23–49	7.44	7.44	
291	6–13–49	7.26	7.97	7.97
	6–23–49	8.67		
84	7–12–46	15.02	15.02	12.94
	6–10–49	8.79	8.79	
General mean				12.88

TABLE V. Mean Values and Number of Occasions for the Determination of p-aminohippurate and Penicillin—G "Filtration Fractions" for Each Dog.

Dog*	A F.F. (PAH)	B F.F. penicillin—G	Difference (A)–(B)
1	.46 (8)	.34 (31)	.12
2	.41(24)	.39 (16)	.02
3	.36 (1)	.30 (5)	.06
4	.41 (1)	.42 (42)	–.01
5	.43 (2)	.35 (50)	.08
6	.31 (3)	.32 (55)	–.01
7	.44 (3)	.32 (60)	.12
8	.42 (1)	.24 (1)	.18
9	.37 (9)	.35 (5)	.02
10	.37(11)	.36 (5)	.01
Weighted mean	.40(63)	.35(270)	.05

* Dog numbers are tabular and are not necessarily the same for each animal from table to table.

Comment. Since so few data, except representative experiments, appear in the literature we have felt it justifiable to present this analysis of "normal" values for canine renal functions despite its limitations. The limitations imposed on this study derive principally from the fact that the data used were the control values from experiments designed for other purposes. Perhaps the statistical analysis has suffered from the limitation of unequal numbers of values and what a statistician would consider an unorderly sequence of accumulation.

The analysis of creatinine glomerular filtration (GF) rate is the most reliable because of the large number (2,045) of accurate determinations on the greatest population. Even so, there were insufficient data over a long enough span of the animals' lives to indicate a trend to any change in GF within a 3- to 7-year span.

It was surprising that the filtration fraction (FF) as represented by GF/RPF_{PAH} determined simultaneously should be so high (0.40), as compared to an FF of 0.19(13) for man. Indeed, we customarily found the clearance of penicillin-G at concentrations of 2 units/ml of plasma to exceed or equal the apparent RPF for PAH measured at different times in the same animals. It may be that the values for RPF were influenced by our customary oral administration of PAH. Poor gastrointestinal absorption was made use of to maintain a quite constant PAH plasma concentration of the order of 1.0 mg/100 ml or less following the administration of the drug *per os.* However, recent reports have suggested that in man RPF values were lower when PAH was administered orally than when it was injected parenterally(14).

It is tenable in the absence of suitable evidence that the small portion of PAH absorbed is either partially hydrolyzed to p-aminobenzoic acid and glycine or conjugated as the

glucuronide within the intestine on the portal system. Either of the latter p-amino compounds would be incorporated into the analysis for PAH and would decrease the apparent clearance of that compound. Checks of various lots of PAH from our source have yielded no evidence that the material administered was contaminated with p-aminobenzoic acid.

In spite of the greater inherent error in the microbiological assay, the "filtration fraction" for simultaneously determined creatinine and penicillin-G clearances is probably the more reliable figure, although that agent is not commonly employed for the measurement of renal plasma flow. Penicillin-G does have the advantage, though, that seemingly large variations in microbiologically active plasma concentrations have no effect on its clearance.

While the number of Tm_{PAH} determinations were few they indicated a satisfactory order of reproducibility of the values for any animal within an experiment, from day to day and for a population of apparently normal dogs.

The Tm values for arginine were found to be as reproducible within repeated tests in a given experiment or from day to day as obtained for PAH. However, the variation of the values from year to year was evident for each dog where comparisons could be made. Although this fluctuation is strictly unaccountable, it may be thought to be due to subtle changes in the microbiological assay of the compound from time to time.

Lastly, it should be pointed out that these data all represent values for the female. Indeed, there seem to be insufficient information in the literature to judge whether systematic sex differences in these functions obtain in the dog.

Summary. From data accumulated over a 6-year period on 31 trained, unanesthetized female dogs the following mean values for several renal functions were calculated: glomerular filtration rate (creatinine clearance) = 94 ml/min./sq. M surface area; renal plasma flow (p-aminohippurate clearance following its oral administration) = 238 ml/min./sq. M; p-aminohippurate Tm = 16.5 mg/min./sq. M; arginine Tm = 12.9 mg/min./sq. M; simultaneous (creatinine) GF/p-aminohippurate RPF filtration fraction = 0.40; and GF/penicillin RPF "filtration fraction" = 0.35. A statistical analysis of the variability of the results was presented and the significance of the data was discussed.

The authors wish to acknowledge the indispensable cooperation of Dr. Lemuel D. Wright and Mrs. Helen R. Skeggs and their associates for the microbiological arginine analyses; of Drs. W. F. Verwey and A. Katherine Miller and their associates for the penicillin assays; and of Miss Elizabeth K. Tillson and her colleagues for the creatinine and p-aminohippurate analyses.

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Received July 7, 1952. P.S.E.B.M., 1952, v80.

Preparation of Radioactive Iodinated Serum Albumin.* (19747)

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The preparation of proteins tagged with Iodine-131 has been described by many workers(1-4). In order to use these preparations for dilution experiments, exhaustive dialysis is necessary to free the tagged protein from free radioactive iodide.

A more rapid method for this preparation has been developed which eliminates the lengthy dialysis step. Ion exchange resins have been used elsewhere in the de-salting of solutions. The studies reported here indicate that the Amberlite resins IR-100H and IR-4B may be used to advantage for the preparation of radioactive iodinated serum albumin solutions free from inorganic iodide.

Preparation. In a 125 ml Erlenmeyer flask, 20 ml of salt-free human serum albumin (25% solution as supplied by the American Red Cross) were mixed with 5 ml of sodium carbonate solution containing 25 g Na_2CO_3 per 100 ml. The iodinating mixture was prepared in a dropping funnel by adding 1.2 of 0.002 M sodium iodide solution and 1.2 ml of 0.02 M sodium nitrite solution to 1.0 ml of a solution of carrier-free Iodine-131 in sodium bisulfite, as supplied by Oak Ridge, with an activity of approximately 1 millicurie per ml. The solution was stirred and brought to a pH below 4 (indicator paper) by the addition of 3 drops of 0.5 N hydrochloric acid. After a few minutes, the solution was neutralized with 2 drops of 0.25 N sodium hydroxide solution. The reagent was diluted with 16.5 ml of water, stirred, and added dropwise to the alkaline albumin solution with gentle stirring over a period of 5 minutes. The funnel was rinsed with 5 ml of water, which were added to the reaction mixture. After 10 minutes of further stirring, the protein solution was poured onto a column of Amberlite IR-4B (analytical grade, previously backwashed with distilled water for 30 minutes). The effluent was then passed through a second column containing backwashed analytical grade Amberlite IR-100H. The first 20 ml of the effluent was made isotonic by the addition of 1.3 ml of 15% sodium chloride solution and filtered through a sterile Seitz-Manteufel filter into a sterile tube. The tube was stoppered with a sterile rubber dam and stored in the refrigerator until needed. The total time elapsed in the preparation was usually about 2 hours.

Efficiency of column-desalting. To determine the amount of inorganic iodide not removed by the column, the following experiment was routinely performed. One ml of the preparation was deproteinized with 1 ml of 10% sodium tungstate solution and 8 ml of N/12 sulfuric acid. The filtrate was counted, with an end-window type Geiger tube, as was a 1:10 dilution of the protein solution. The following results are typical:

1:10 albumin solution: 25,893 $\pm$ 2% cpm

Deproteinized solution 323 $\pm$ 2% cpm

It is apparent that virtually all of the non-protein-bound iodine has been removed by the deionization procedure.

Summary. A rapid procedure for the preparation of radioactive iodinated human serum albumin suitable for injection for dilution studies has been described. This technic used ion exchange resins instead of dialysis for the removal of free inorganic iodide.

* This research was done under the auspices of the Atomic Energy Commission.

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Received July 10, 1952. P.S.E.B.M., 1952, v80.

Biochemical Studies on the Lizard, *Anolis carolinensis.* (19748)

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Although those mammals of interest to biochemistry have been studied in detail for some 50 years, comparative biochemistry as a field of study is little more advanced than was the field of comparative anatomy a hundred years ago. It is curious to note that a century old knowledge of the anatomy of most of the common reptiles has not stimulated research in the chemical composition and biochemistry of this important class of animals.

Among reptiles the "American chameleon," *Anolis carolinensis,* is plentiful, inexpensive and has many other advantages in research requiring the sacrifice of large numbers of animals. Before this animal could be used for biochemical research, a knowledge of the composition of the blood, urine, and ash was thought to be necessary. Since *Anolis carolinensis* when fully grown weighs only 5 to 7 g, micro and ultramicro analytical technics were required in this survey study.

All analyses were made on recently captured animals which were fasted 4 days before use. Blood for glucose determinations was obtained by decapitation; blood for all other analyses was obtained by cardiac puncture in the following manner: About 0.001 ml of a 0.004% solution of heparin was added to the tip of a tuberculin syringe fitted with a No. 26 needle. While the animal was held ventral surface up on a table by an assistant, the needle of the syringe was placed just anterior to the pectoral girdle in the midline, parallel to the body of the animal and pointed toward its tail. The needle was then pushed carefully through the skin into the region just beneath the pectoral girdle. With careful probing the heart was penetrated, and following slight suction on the syringe, blood was obtained. From a single animal one can obtain a maximum of 0.05 to 0.20 ml of blood. If less than one-half of the total estimated blood volume is drawn, 7 out of 10 animals will survive such a blood letting.

If blood pH was to be determined or if whole blood was required, the capillary tube used in pH determinations, and the blood pipettes were filled directly from the blood in the syringe. If plasma was required, a metal clamp was fitted to the syringe between the head of the plunger and the body of the syringe and the blood was then centrifuged in the syringe. If acid base studies were to be done, a drop of mineral oil was layered above the blood. After centrifuging the syringe was held vertically and the plunger carefully twisted upward. By this technic practically all of the 0.03 to 0.15 ml of plasma was measured directly into blood pipettes for analyses without disturbing the packed cell layer in the syringe. Using such methods and ultramicroanalysis pH, Na, K, Cl and CO_2 could be done on the plasma of one animal.

Urine was obtained by exerting slight pressure on the abdomen just anterior to the anus and collecting the drops of urine in measuring pipettes. Using this method 0.01 to 0.20 ml of urine was obtained per animal.

Ash studies were carried out in November, on animals recently caught. After a 4-day fast they were weighed, decapitated, dried at 75°C to constant weight, and ashed in a muffle furnace at 450-500°C. The resulting ash was weighed, ground to a very fine powder, placed in a 10 ml volumetric flask, diluted to mark with distilled water, stirred thoroughly and allowed to settle. The supernatant fluid was analyzed then for Cl, Na and K. Following the analyses of these water-soluble constituents, enough HCl was added to the flask to dissolve the water-insoluble Ca and Mg salts. Water was added to mark and samples of the acid solution were analyzed for Ca and Mg.

Analytical methods. Packed cell volume determined by the method of van Allen(1). Na and K were analyzed with the Beckman flame spectrophotometer. Chlorides were determined in plasma, urine and protein-free filtrates of blood by the method of Schales and Schales(2) adapted to small volumes of sample through the use of the ultramicro

TABLE I. Blood Constituents.

		Avg	Analyses	Range
Packed cell vol (%)		28	26	20 – 34
Red blood cells (10^6/mm^3)		.96	26	.61– 1.21
Oxygen capacity (vol %)		9.3	15	7 – 12.5
Plasma	pH	7.26	25	6.93– 7.63
	Na (mM/L)	157	41	139 –186
	K "	4.59	15	2.80– 5.93
	Cl "	127	30	113 –133
	CO_2 "	15.4	25	9.6 – 22.5
	Ca "	2.9	20	2 – 4.2
	P "	2.6	19	1.7 – 3.2
	Protein (%)	4.1	12	3 – 5.7
Blood	Na (mM/L)	130	15	118 –135
	K "	31.8	15	20.9 – 38.4
	Cl "	105	18	78 –116
	CO_2 "	14	15	9.3 – 17.1
	Protein (%)	11.5	16	9.1 – 13.6
	Uric acid (mg %)	7.9	20	4.1 – 10.8
	Urea "	7.2	15	5.5 – 9.8
	NH_3 "	Tr	15	0 – .9
	Glucose "	172	715	58 –305

TABLE II. Urine Constituents.

	Avg	Analyses	Range
pH	5.54	19	4.62– 6.50
Na (mM/L)	20.2	18	5.7 – 33.7
K "	17.1	18	5.1 – 36.9
Cl "	23.4	22	4 – 49
CO_2 "	Tr	15	0 – 1
Uric acid N (mg %)	45.6	19	5.3 –166.5
Urea N "	8.8	20	4.9 – 18.5
Ammonia N "	8.2	20	4.9 – 22.8
Creatinine N "	1.3	18	.6 – 1.9

burette of Gilmont(3) and technics of microanalysis suggested by Kirk(4). CO_2 and O_2 capacity were measured in the Scholander syringe analyzer(5,6). The Beckman model G pH meter fitted with the Beckman No. 290-83 capillary glass electrode was used to determine the pH of blood and urine. Plasma Ca was determined by the method of Sobel and Sobel(7), plasma P by the method of Kuttner and Cohen(8). Ash Ca was determined by permanganate titration(9), Mg by the method of Denis(10). Blood and plasma were digested and fractions of the digest were added to Conway diffusion cells and the protein determined as ammonia(11). Ammonia, urea and ash Cl also were analyzed in Conway cells(11). Blood uric acid was determined by the method of Brown(12), urine uric acid by the method of Folin(13). Creatinine in the urine was measured by the method of Folin and Wu(14). A micro adaptation of the method of Folin and Wu(15) was used to determine blood glucose.

The results of these analyses appear in the accompanying tables. Compared to the alligator(16) and turtles(17), *Anolis* has a higher blood Cl and lower pH. Inorganic P is high as compared to the alligator and human. The distribution of body water may be approximated from the average values of Cl in the ash and plasma on the assumption that all Cl is extracellular. In the average animal in November intracellular fluid is 44.7% and extracellular fluid 22.7% body weight.

Blood glucose averages 172 mg % over the year but there is a seasonal variation, an average minimum of 110 mg % in August, average maximum of 197 mg % in February. There are no sex differences. In order to check the high values of blood sugar by a more specific method, a series of fermentation studies were carried out using a yeast microdiffusion method in a special Conway cell(18). Fermentable sugar averaged 159 mg % as compared with the value of 186 mg % obtained on a control series using the Folin and Wu method.

Summary. The following determinations have been done on freshly caught fasting specimens of *Anolis carolinensis*: red cell count, hematocrit, O_2 capacity, blood glucose, blood

TABLE III. Composition of Anolis Ash.

Sex	Wet wt	Dry wt	Ash wt	Na	K	Ca	Mg	Cl
	g			mg				
♂	3.50	1.32	.175	3.35	5.87	58.3	1.34	3.85
♂	3.26	.99	.135	2.86	5.23	43.2	1.10	2.73
♂	3.02	.90	.132	2.23	5.03	45.6	1.19	3.30
♂	3.71	1.26	.173	2.39	6.23	57.9	1.30	4.20
♀	3.22	1.00	.153	2.80	5.65	51.6	1.39	3.12

and urine urea, ammonia, uric acid, Cl, Na, K, pH, CO_2; blood and plasma proteins; urine creatinine, and Na, K, Ca, Mg and Cl of the ashed animal.

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Received July 10, 1952. P.S.E.B.M., 1952, v80.

Effects of Liver Injury and Nephrectomy on the Anticonvulsant Activity of Phenacemide.*† (19749)

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Although phenacemide (phenacetylurea; Phenurone) is one of the most useful agents for the treatment of psychomotor epilepsy, comparatively little is known concerning the fate and the excretion of this agent in animals or man. In a brief report on the pharmacology of Phenurone, Everett(1) mentioned experiments in which the duration of anticonvulsant action remained unchanged in bilaterally nephrectomized mice, but was significantly prolonged by carbon tetrachloride-induced liver damage and hepatectomy. We are aware of no published information concerning the fate and excretion of Phenurone in man.

We have reported(2,3) the results of experiments in which biological methods were used to measure the effect of liver injury and nephrectomy on the potency and the duration of anticonvulsant action of clinically employed hydantoins and oxazolidine-2,4-diones. We have now applied the same biological methods in the study of Phenurone. It was anticipated that the results would contribute information

* This investigation was supported by research grants from the National Institutes of Health, Public Health Service, and the University of Utah Research Fund.

† Phenacemide was kindly supplied by Dr. R. K. Richards, Abbott Laboratories.

on the relative importance of the liver and the kidney for the fate and the excretion of this compound, as interpreted from the alterations in potency and duration of action induced by the experimental conditions employed.

Methods. Adult male albino rats obtained from the Sprague-Dawley or Holtzman-Rolfsmeyer farms were used as experimental animals. They were maintained on an adequate diet and allowed free access to food and water except during experimental testing. Because of its insolubility, Phenurone was given orally as a 0.5 or 1.0% suspension in a 10% acacia solution. Anticonvulsant potency, as measured by the maximal electroshock seizure test, was determined in normal, nephrectomized and liver-injured rats at 3, 6 and 12 hours after administration of the drug. The details of the maximal electroshock seizure test have been described elsewhere(4). Briefly, it consists in determining the dose of a drug which abolishes the hindleg tonic extensor component of the maximal electroshock seizure pattern in 50% of animals. The ED_{50}s were calculated by the method of Litchfield and Wilcoxon(5). From 14 to 72 animals were used for the determination of each ED_{50}. Liver injury was induced by a single subcutaneous injection of 2 ml/kg of a 50% (w/v) solution of carbon tetrachloride in peanut oil, and the drug was administered 36 to 48 hours later. Histological studies of liver sections taken from rats subjected to such carbon tetrachloride treatment showed that moderate to severe liver damage was consistently produced. Nephrectomy was performed under ether anesthesia at such a time interval before drug administration that 12 hours always elapsed between time of operation and time of electroshock test; in this manner, the effect of accumulated metabolic products was kept relatively constant. All animals (normal, nephrectomized and liver-injured) were previously examined for their ability to exhibit a maximal electroshock seizure pattern. In all instances they were found able to respond with a normal pattern; thus nephrectomy and liver injury *per se* did not alter the response to electroshock. Therefore, any changes observed could reasonably be attributed to Phenurone.

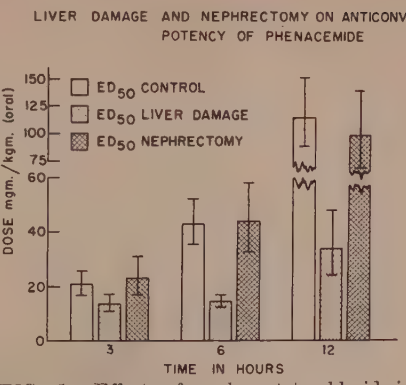

FIG. 1. Effect of carbon tetrachloride-induced liver injury and of nephrectomy on anticonvulsant potency of phenacemide. Anticonvulsant doses are expressed in mg/kg along the ordinate and the time intervals at which tests were made after drug administration are shown on the abscissa. The significance of the various vertical bars is indicated by the legend in the figure. The 95% confidence limits are shown by the bracketed vertical lines.

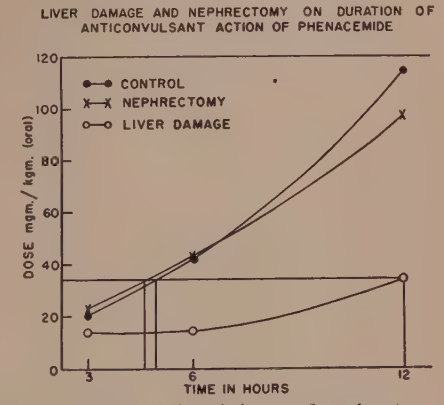

FIG. 2. Effect of liver injury and nephrectomy on duration of anticonvulsant action of phenacemide. See text for explanation.

Results. The effects of nephrectomy and of liver injury on the anticonvulsant *potency* of Phenurone are shown in Fig. 1. It may be seen from Fig. 1 that liver injury tends to reduce significantly the dose of Phenurone required to modify the maximal electroshock seizure pattern in 50% of rats at all 3 time intervals studied. For example, a dose of 115 mg/kg of Phenurone was required to protect 50% of normal rats at the 12-hour interval, as compared with a dose of only 34 mg/kg in rats with damaged livers [potency ratio with

95% confidence limits = 3.4 (2.2 to 5.2)]. In contrast, bilateral nephrectomy has no statistically significant effect on the anticonvulsant potency of Phenurone. With regard to duration of action, pertinent information was obtained by plotting the ED_{50}s against time, as shown in Fig. 2. In a recent publication(2) duration of anticonvulsant action was calculated indirectly rather than by the direct graphic method employed herein. The 2 methods give essentially the same results but the graphic technic is easier and more readily comprehended. In Fig. 2 the smooth curves which join the observed values were fitted by eye. A horizontal line was drawn to intercept the ordinate at the 34 mg/kg dose level. Where this line crosses the dose-time curves, perpendicular lines were drawn to the abscissa. From an inspection of this plot of the data, it can be seen that a dose of 34 mg/kg of Phenurone protects at the 50% level for less than 5 hours in normal rats and for slightly over 4½ hours in nephrectomized animals. In sharp contrast, the same dose of Phenurone protects liver-injured rats for 12 hours. It is thus evident that liver injury, but not nephrectomy, significantly increases the duration of anticonvulsant action of Phenurone.

Discussion. The data presented substantiate the observation of Everett(1) that carbon tetrachloride-induced liver injury, but not nephrectomy, increases the duration of anticonvulsant action of Phenurone. In addition, carbon tetrachloride-induced liver injury significantly increases the anticonvulsant potency of Phenurone at all time intervals studied; the average increase in potency was approximately 250%. In contrast, bilateral nephrectomy had no significant effect on the anticonvulsant potency or the duration of action of Phenurone.

The fact that hepatic damage increases both potency and duration of action of Phenurone can be interpreted to mean that the liver is important for the degradation of Phenurone into substances devoid of anticonvulsant activity as measured by the maximal electroshock seizure test. The observation that bilateral nephrectomy neither increases anticonvulsant potency nor prolongs the duration of action of Phenurone suggests that the kidney is not the principal organ for the elimination of this drug or any active product into which it may be converted.

Summary. Phenurone was tested at various time intervals after administration in normal, liver-injured and nephrectomized rats for its ability to prevent the tonic extensor component of maximal electroshock seizures. The results were analyzed for the effect of liver injury and nephrectomy on the potency and the duration of anticonvulsant action of this compound. Liver injury significantly increased the potency and the duration of anticonvulsant action; on the other hand, bilateral nephrectomy had no significant effect on the anticonvulsant properties of this agent. The results suggest that, in the rat, the liver is the principal organ for the degradation of Phenurone into products devoid of anticonvulsant action, and that the kidney plays no important role in the metabolic alteration or excretion of this compound.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Morning Changes in Number of Circulating Eosinophils in Infants.* (19750)

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Human infants exhibit cycles in bodily activity of about 3, rather than of 24-hour duration(1,2). The daily temperature curve shows only slight deviations from a straight line during the neonatal period(3,4). The 24-hour rhythm in the glycogen content of the liver of rats is absent during the first 2 weeks of life—a period roughly corresponding for man to that covered by the last months of intrauterine life and the first 2 months after birth(5). These observations suggest that several 24-hour periodicities which are features of the physiology of mammals from adolescence throughout senescence are absent during the neonatal period. Accordingly, an inquiry into the time of appearance of the 24-hour rhythm in number of eosinophils, which has been established for mature mice(6) and man(7), appeared to be of possible interest. This was carried out by means of endogenous eosinopenia tests(8) on infants of 2 age groups.

Materials and methods. The subjects of this investigation were patients of the pediatric service at the University of Minnesota Hospitals convalescing from non-endocrine disease. Infants of both sexes constituted the 2 age groups listed in Table I. Nursing of Group I infants was carried out unchanged on the morning of testing at 4-hour intervals starting daily at 01:30, according to the routine of this hospital. The infants constituting Group II had breakfast at 07:30. Stimulation other than daily routine was avoided for all the subjects of this investigation. The time of day of blood sampling was kept identical with the schedule suggested for endogenous-eosinopenia tests in adults(8). Samples of capillary blood were obtained at 06:30 and at 09:30 of the same morning for eosinophil counts by the direct chamber method. Certified diluting pipettes, Randolph's diluting fluid(9), and certified 0.2 MM deep Levy counting chambers with Fuchs-Rosenthal double ruling were employed.

TABLE I. Summary of Morning Changes in Number of Eosinophils per mm^3 in 2 Age Groups of Children.

Group No.	Chronological age	No. of tests	Mean* ± S.E.	t	P_t
1	1 day to 7 mo	16	+54±24	2.21	.043
2	Over 15 mo	17	−39±12	3.32	.005

* Mean % change from the 06:30 to the 09:30 count (with the value obtained at 06:30 equal to 100%).

Results. The results are expressed as per cent change from the 06:30 to the 09:30 count, with the value obtained at 06:30 equal to 100%. A considerable scatter in the morning change in eosinophils in the infants composing the 2 groups studied is readily apparent from Fig. 1. However, most of the infants older than 15 months (Group II subjects) show a decrease in number of circulating eosinophils, a phenomenon established by earlier work for mature man(7). It can be seen from Table I that the endogenous eosinopenia of Group II subjects is significant below the 1% level ($P = .005$). By contrast, only 4 out of a total of 16 tests on Group I infants show a decrease in number of eosinophils. These infants show on the average an increase (+ 54%; $P = .043$). The difference in mean per cent change in number of eosinophils from 06:30 to 09:30 for the 2 groups investigated is 93 ± 27 ($P = .001$). It appears fair to infer that we deal with 2 different populations of infants, from the point of view of their variations in number of eosinophils.

Discussion. The absence of endogenous eosinopenia from 06:30 to 09:30 in Group I subjects could possibly indicate that eosinophil variations during early infancy lack the 24-hour spacing noted later in life. The data indicate that tests for endogenous eosinopenia which employ the 06:30 to 09:30 sampling schedule can be carried out from the age of

* This investigation was supported in part by a Research Grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† The assistance of Charles Stewart, M.D., is appreciated.

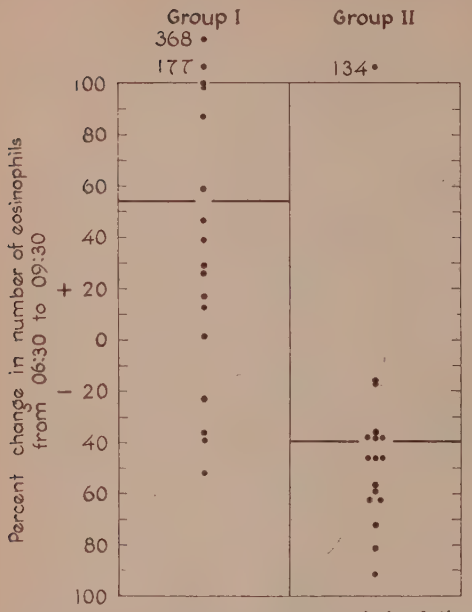

FIG. 1. Morning changes in number of circulating eosinophils in 2 groups of infants (Group 1: younger than 7 months; Group 2: older than 15 months).

15 months onward with the same results as in adults. Tests during the first 7 months of life yield predominantly positive results, *i.e.*, an increase rather than a decrease in number of eosinophils.

It has been shown that endogenous eosinopenia in mature man is synchronized with initiation of daily activities(10). It is conceivable that the several alternations of the activity (and/or feeding) cycle within 24-hour periods, in infants, are associated with eosinophil variations which likewise occur over shorter periods. The marked endogenous eosinopenia in Group II infants is in keeping with the assumption of a developed 24-hour rhythm in number of eosinophils in infants older than 15 months. In view of the parallelisms between the activity and eosinophil rhythms it is of further interest that the activity rhythm of a baby, studied during the first 15 months of its life by Mullin, showed a steady rise in the day to night ratio for bodily activity up to the 7th month(11). It may be also of interest that marked 24-hour periodicity in body temperature of infants first appears during the second year of life, although an approach to a temperature rhythm can be discerned during the first year in that 24-hour variations gradually increase in amplitude (12). Finally, it is noted that the 24-hour rhythm in the glycogen content of the liver in rats develops during the 3rd week of life—a period roughly corresponding to that covered by about the 3rd to the 9th month of human postnatal life(5).

Summary. Morning changes in number of eosinophils were studied in 2 age groups of infants, in the absence of stimulation other than daily routine. Endogenous morning eosopenia, which is one of several physiological periodicities exhibiting 24-hour spacing in maturity, is not revealed in early infancy by tests employing the 06:30-09:30 sampling. By contrast, most infants older than 15 months show a marked decrease in number of eosinophils from 06:30 to 09:30 of the same morning. The significance of these findings is discussed.

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Received July 14, 1952. P.S.E.B.M., 1952, v80.

Studies on Copper Metabolism. V. Storage of Iron in Liver of Copper-Deficient Rats.* (19751)

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It has been demonstrated in previous studies in this laboratory(1,2) that swine depleted of copper develop an anemia which is morphologically indistinguishable from iron-deficiency anemia and which is accompanied by hypoferremia and an increase in the iron-binding capacity of the plasma. Evidence has been presented that in such animals there is 1) impaired ability to absorb iron from the gastro-intestinal tract; 2) incomplete mobilization of iron from the tissues; and 3) an inability to utilize parenterally administered iron for hemoglobin synthesis even when it is presented to the bone marrow in normal quantities. The fact that copper influences iron metabolism in such diverse sites as the mucosal cell, the liver, and the bone marrow, suggests that this element may, in some basic manner, be concerned wherever and whenever iron moves. Since ferritin has been shown to be present in the mucosal cell, the liver and the bone marrow(3) it seems plausible that copper may be concerned with the turnover of iron through ferritin.

In a previous study in swine(2) the iron in the livers and spleens of control, copper-deficient and iron-deficient pigs, and copper-deficient pigs given 200 mg of iron intravenously, was fractionated into the saline-soluble and the saline-insoluble portions. It has been shown by others(3-5) that the saline-soluble fraction contains most of the ferritin and that hemosiderin iron can be extracted with dilute hydrochloric acid from the residue remaining after saline extraction. The results of this preliminary study suggested that after the intravenous injection of iron into copper-deficient swine there was a tendency to store more iron as "hemosiderin" and less as "ferritin" as compared with control animals with approximately the same concentration of total iron in the livers. However, the results of this study were inconclusive.

The purpose of the present report is to present studies on the rate of uptake of parenterally administered radioactive iron into the saline-soluble (presumably ferritin) and saline-insoluble (presumably hemosiderin) fractions of the livers of control and of copper-deficient albino rats.

Methods. A total of 81 male weanling rats of the Sprague-Dawley strain were used. All of the rats were fed a diet of commercial canned milk diluted 1:1 with distilled water‡ supplemented with one mg of iron§ as ferrous chloride 3 times a week. A control group of 27 rats received, in addition, 0.1 mg of copper/rat as copper sulfate 3 times a week. These supplements were offered in a small quantity of milk in the morning to insure their complete ingestion. The amount of diet fed the control rats was restricted to the amount eaten by the deficient rats.

Two groups of copper-deficient rats were studied. When the mean hemoglobin level had decreased to 11.5 g %, the rats in the first copper-deficient group were given one ml of an iron solution buffered with 3% sodium citrate containing 0.04 μc of Fe^{59}‖ activity and 200 μg of total iron by the subcutaneous route. At the same time the control animals were given the same amount of radioiron. The animals in the second copper-deficient group were allowed to continue on the low-copper diet until the mean hemoglobin level reached 9.0 g %. At this time, the animals were given isotopic iron in the same amount as above. One-third of each group of rats was anes-

* Aided by a grant from the U. S. Atomic Energy Commission, Contract No. AT (11-1)-82.

† A.E.C. Post-doctoral Fellow in the Medical Sciences (1949-1952).

‡ Containing 0.05 to 0.09 mg of copper/liter by analysis after dilution.

§ Carbonyl iron powder, grade RX, obtained from Antara Products, General Aniline and Film Corp.

‖ Obtained on allocation from the U. S. Atomic Energy Commission.

TABLE I. Partition of Subcutaneously Injected Radioiron Between Saline-Soluble (Ferritin) and Saline-Insoluble (Hemosiderin) Fractions of Liver from Control and Copper-Deficient Rats. Results expressed in μg of iron per total liver fraction $\pm$ the S.E. (standard error) of the mean.

Group	No. of rats	Fraction	Time after inj, hr 8	24	48
Control	27	Saline-soluble	50 $\pm$ 2.8	43 $\pm$ 2	42 $\pm$ 4.7
		Saline-insoluble	9 $\pm$ .6	10 $\pm$ .3	16 $\pm$ 1.3
Copper-deficient I	27	Saline-soluble	32 $\pm$ 2.1	32 $\pm$ 2.1	33 $\pm$ 2.7
		Saline-insoluble	8 $\pm$ .7	9 $\pm$ .7	11 $\pm$ .9
Copper-deficient II	27	Saline-soluble	52 $\pm$ 7.2	46 $\pm$ 6.1	49 $\pm$ 8.3
		Saline-insoluble	9 $\pm$ 1.4	7 $\pm$ 1.1	9 $\pm$ 1.3

thetized with ether and sacrificed by hemorrhaging at 8, 24 and 48 hours after the injection of iron. The livers were removed, blotted free of blood with gauze and weighed. The whole livers were then homogenized in a Waring blendor for 2½ minutes with sufficient 0.9% saline to make a total volume of 50 ml. A 15 ml aliquot was pipetted into 2 small (10 mm) test tubes and centrifuged at 2200 x g for one half hour. The supernatant fluids from both tubes were transferred to a 100 ml pyrex centrifuge tube. The insoluble residues were mixed with approximately 15 ml of 0.9% saline and transferred to one small test tube and centrifuged. The supernatant fluid was added to the first 2 supernates in the 100 ml centrifuge tube. The combined supernatant solutions were heated at 75°C for 15 minutes. The precipitated hemoglobin was removed by centrifugation and discarded. The supernatant solution was transferred to a second 100 ml centrifuge tube containing 10 to 15 ml of 0.9% saline and heated for 20 minutes in a boiling water bath. The precipitate, representing the saline-soluble or "ferritin" fraction was then separated by centrifugation, resuspended in saline, transferred to a 10 mm test tube, and recentrifuged.

The radioactivity in the saline-soluble fraction and in the original saline-insoluble residue was measured in a vial sample scintillation counter as described previously(6). A standard containing 10% of the injected amount of radioiron was counted along with each group of samples. By comparison of the radioactivity in the liver fractions with the standard, the iron represented by the total counts was calculated.

Results and discussion. The results are presented in Table I. Although the same total amount of iron did not reach the liver in all 3 groups, in all groups 80 to 85% of the radioiron taken up by the liver was found in the saline-soluble (ferritin) fraction within 8 hours. Fifteen to 20% of the iron reaching the liver was present in the saline-insoluble (hemosiderin) fraction. No significant difference, either in the partition of the iron after 2 days or in the rate of uptake of the iron into the 2 fractions was observed between the control and the copper-deficient animals. Likewise no significant differences were observed between the more severely (Group II) copper-deficient and the less severely (Group I) copper-deficient rats.

Since no studies were made on the efficiency of the method used in separating ferritin from hemosiderin and since these compounds were not identified other than on the basis of solubility, it cannot be stated conclusively that the rate of ferritin synthesis was unaltered. However, the results suggest that the derangement in iron metabolism in copper-deficient rats is not a consequence of an abnormality in the manner of storage of iron in the liver.

Summary. No alteration from the normal in the uptake of parenterally administered radioiron into the saline-soluble and saline-insoluble fractions of liver iron was observed in copper-deficient albino rats.

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Received July 16, 1952. P.S.E.B.M., 1952, v80.

Serum Lipids Studied by Electrophoresis on Paper. (19752)

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Several lines of investigation have indicated the lipids of human serum and plasma to be associated with proteins. Analysis of electrophoretically separated protein fractions of serum has revealed the presence of lipid in all(1), the highest concentration being in the β-globulin fraction(1-4). Nearly all the plasma cholesterol and phospholipid may be recovered in Cohn fractions III_0, IV, V, and VI(5) and in pooled plasma an α and a β lipoprotein have been described, the latter containing 75% of the total plasma cholesterol(6). A large number of lipoprotein classes has been disclosed by ultracentrifugation of plasma(7).

Microelectrophoresis of proteins on filter paper(8) has proved to be a useful method for the separation and study of serum proteins(8-11). In the present study histochemical and other procedures applicable to the detection and characterization of lipids in tissue sections have been applied to paper strips on which electrophoresis of human serum samples had been performed and the lipid and protein patterns were compared. A brief communication describing a similar approach to the study of blood lipoproteins has recently been published(12).

Methods. The procedure used for electrophoresis was, with minor modifications, that described by Durrum(8). The sera studied were obtained from 74 individuals and included both normal subjects and hospitalized patients suffering from various disorders. The serum sample (0.1-0.2 cc) was applied transversely as a band approximately 1 cm wide across a 42 x 11 cm piece of Whatman 3 MM paper and the paper adjacent to the band was then quickly moistened with sodium barbital buffer (0.025 M, pH 8.6). The paper was then placed in a lucite-covered glass aquarium so that the middle of the paper rested on a silicone-coated glass rod traversing the aquarium 20 cm above its floor and the ends (6 cm apart) dipped into buffer-containing electrode vessels in which were also immersed platinum electrodes connected, via mercury-filled glass tubes, to lead wires from the power supply. The paper on either side of the serum line was then drenched thoroughly with buffer and voltage applied. The line of application of the serum was generally 4.5 cm from the apex on the cathode side; in this position the γ globulins moved very little, their migration toward the anode being very nearly balanced by the electro-osmotic flow of liquid toward the cathode. If the line of origin was more toward the cathode there was a greater rate of movement of all bands toward the anode but without greater resolution, while if the placement were more toward the anode the components of least mobility moved toward the cathode. Three hundred volts, providing a current of 0.4 milliampere per cm of width, applied for 5 hours moved the front of the fastest band approximately 15 cm from the origin. The paper was then air-dried in a hood, heated for 10 minutes in an oven at 100°C, and, after removal of the outer 0.5 cm on each side, was cut into parallel longitudinal strips 1-2 cm wide to be stained separately. Proteins were stained according to Durrum's method(8) by immersion of a strip in alcoholic bromphenol blue solution saturated with mercuric chloride and subsequent washing in water. For demonstrating lipids another paper strip was immersed in a saturated solution of Sudan IV in 40% ethanol, 50% glycerol

and 10% water; this was prepared (except for the use of Sudan IV instead of III) according to the method of Rossman(13), by mixing equal volumes of glycerol and a saturated solution of the dye in 80% ethanol, and filtering. Papers were allowed to remain in this solution for approximately 16 hours at room temperature, and then were washed thoroughly in water and dried. The areas containing lipid were stained red, standing out against a faintly pink background; the latter was probably not caused by lipid originally in the paper since prolonged extraction of blank paper with petroleum ether prior to staining decreased it only slightly. The Sudan stain described gave better results than Sudan IV solution in 70% ethanol, 35% ethanol-50% acetone, and Sudan II in isopropanol (60%). Exposure of paper to osmium tetroxide vapors was also unsatisfactory as a method for fat demonstration, the method lacking sensitivity and specificity. The Schultz test(14), embodying the Lieberman-Burchard reaction, was modified for use under these conditions, as a means for demonstrating cholesterol in the paper. A paper strip was soaked in aqueous 20% ferric chloride solution for 15 minutes(15), washed thoroughly in water, rinsed in 2 N hydrochloric acid, and again in water, and dried in the oven. The paper was then laid on a glass plate over a white background, a solution of 1:1 glacial acetic-concentrated sulfuric acid applied with a pipette, and another glass plate was quickly superimposed; the presence of cholesterol was indicated by a greenish or black color developing after 10-20 minutes, well before the paper began to disintegrate in the strongly acid reagent (in about one hour). Pretreatment of the strip with the ferric chloride solution seemed to give better results than immersion in aqueous hydrogen peroxide or iodic acid. A spray of antimony pentachloride in chloroform(16) was also used as a cholesterol reagent in some instances, but the instability and non-specificity of this reagent made it less useful.

The relative quantities of protein and of sudanophilic lipid in different zones of the electrophoretograms were estimated by a densitometric procedure. The papers stained with bromphenol blue and Sudan IV, after drying, were rendered more translucent by applying Krylon Clarifier* with a cloth to one surface of the paper, and then a plastic coating was applied to both surfaces (Krylon Plastic Spray).* The strip thus prepared was passed through the horizontal slit in front of the photocell at the bottom of the cuvette well of the Coleman Junior Spectrophotometer. A metal plate contiguous with the paper and interposed between it and the light source, and provided with a 0.5 cm square aperture permitted the light beam to pass through an equal area of the paper strip, which was moved manually, in 0.5 cm steps, in a vertical direction, the optical density at each point being recorded. For the bromphenol blue papers a wave length of 630 μ was used, and for the Sudan-stained papers 540 μ. The photocell sensitivity was adequate at 630 μ to permit blank setting at O.D. of 0, but at 540 the zero setting was 0.200 or 0.300.

Results. The electrophoretic protein patterns revealed 4 bands which, judged both by comparison with published figures of paper electrophoretic protein patterns(8,10,11) and by relative mobility and apparent quantity, corresponded in order of increasing mobility to the γ, β, α_2 globulins and albumin of free electrophoresis. A discrete band corresponding to α_1 globulin was not observed. It is not clear whether this was attributable to poor resolution or to a failure to demonstrate small quantities of this protein by the dye technic used. Evidence in favor of the latter possibility was provided by radioautographs of paper strips on which electrophoresis had been performed of serum from a hyperthyroid patient who had received a therapeutic dose of I^{131} several days earlier and of normal serum to which labelled thyroxine had been added; in both instances the main radioactive zone was found between the α_2 globulin and albumin, confirming the observation of Gordon and associates(17), who identified this area as corresponding to α_1 globulin. On the other hand, the mobility on paper of a specimen of α_1 glycoprotein(18)† was the same as that of

* Manufactured by Krylon, Inc., 2601 N. Broad St., Philadelphia, Pa.

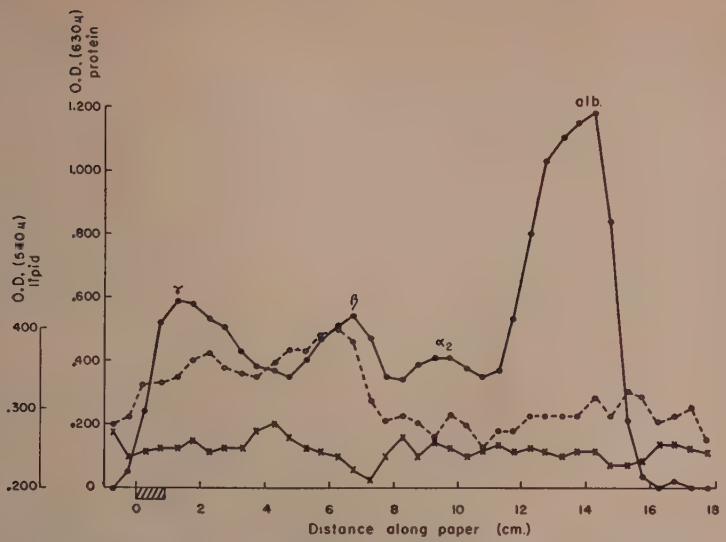

FIG. 1. Electrophoretic pattern of serum of fasting 30-year-old female subject. Optical density is plotted against distance along the paper. The cross-hatched area represents the line of application of the serum. In this and the other figures, solid circles, unbroken line represent protein (bromphenol blue); open circles, broken line, lipid (Sudan). The curve ×——× is the Sudan pattern of a parallel strip of paper extracted with alcohol-ether (3:1) after electrophoresis and prior to staining, and shows nearly complete removal of lipid.

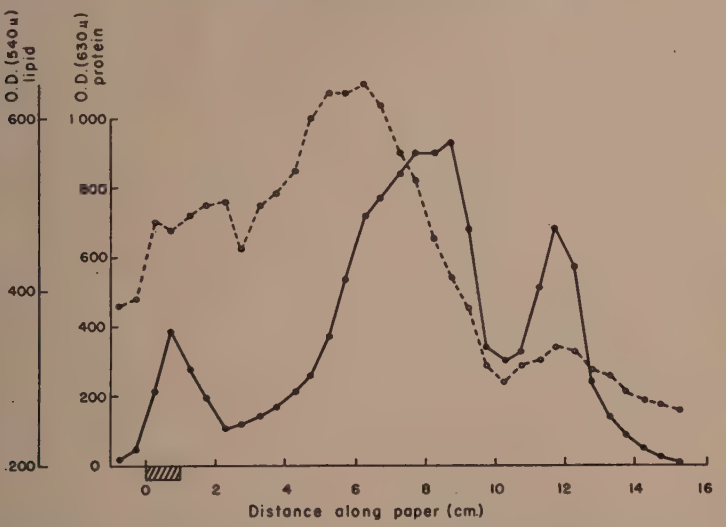

FIG. 2. Electrophoretic pattern of serum from patient with nephrosis and hypercholesterolemia. The figure illustrates the striking decrease in the albumin and γ globulin peaks and the large, fused α and β globulin zone, as well as the great increase in lipid as compared with the serum of Fig. 1.

albumin, and a mixture of these 2 proteins was not resolved, nor did addition of the glycoprotein to serum cause an increased quantity of dye to be retained between α_2 globulin and albumin.

† A specimen was generously supplied by Dr. D. M. Surgenor, Harvard Medical School.

The papers stained with the Sudan dye generally presented 2 distinct red zones, the more prominent corresponding to the β-globulin region and the less intense area to the albumin (Fig. 1). A faint pink trail from the line of application of the serum to the major peak was frequently noted. In some subjects with alimentary lipemia a distinct lipid band was present at the origin; this was not associated with the γ globulins which occupied nearly the same position, since placing the serum sample closer to the anode resulted in migration of the γ globulins toward the cathode while the lipid zone remained at the line of application. This would suggest that this lipid was unassociated with protein. In most serum specimens studied the peak of the main sudanophilic zone was at or close behind that of the β-globulin peak; in 5 subjects the pattern was different, the major Sudan-staining zone extending well beyond the β-globulin and almost reaching the α_2 globulin region.

The Schultz reaction on the papers paralleled, in general, the Sudan-stained papers, the major cholesterol band being in the β-globulin region, with a band occasionally visible at the line of application, and a light trail to the β-globulin zone. The intensity of the darkening produced by the acetic acid-sulfuric acid mixture paralleled the concentration of cholesterol in the serum, very dense black bands being noted in 3 nephrotic sera. In some instances a rather faint darkening in the Schultz test was observed at the albumin area corresponding to the more rapidly moving of the 2 sudanophilic zones, but in many cases it was difficult to be certain of a characteristic color change because of the persistence, even after washing, of a faint brownish color in this region after treatment with ferric chloride. It appeared that a greater proportion of the Schultz-reacting material on the electrophoretogram was in the β-globulin zone, as compared with Sudan-staining material.

No satisfactory histochemical procedure for the direct demonstration of phospholipids was devised. The Baker-Smith-Dietrich method (19) applied to the paper strips stained all 4 protein zones in proportion to the amount of protein in these areas. An indication of the position of phospholipid on the papers was obtained from the electrophoretogram of serum from a patient who had received a therapeutic dose of P^{32} 3 weeks previously.‡ Radioautographs were made of (a) the original strip, (b) a parallel strip after soaking in 5% trichloroacetic acid, and (c) a strip extracted with alcohol:ether (3:1). Radioactivity was present at the β-globulin and albumin areas and at the origin of (a) and of (b), but not (c). This result suggested the presence in these areas of phosphorus-containing substances precipitable with trichloroacetic acid and soluble in fat solvents. Experiments on other sera confirmed this finding. When, prior to Sudan staining, strips were boiled under a reflux for 2 hours in acetone saturated with magnesium chloride, in which phospholipids are relatively insoluble, the intensity of the stain in the β-globulin zone was considerably reduced as compared with unextracted strips, while there was little or no decrease in the sudanophilic lipid at the albumin region. Extraction of the strip with 3:1 alcohol:ether removed virtually all Sudan-staining lipid from both zones, and produced little change in the protein pattern. These findings, together with those in the Schultz test, suggest that there is relatively more cholesterol and less phospholipid in the β-globulin area as compared with the albumin zone, in agreement with chemically determined cholesterol-phospholipid ratios in the β and α lipoproteins (5,20).

The administration of small quantities of heparin is known to induce clearing of the plasma in alimentary lipemia (21) and marked changes in the ultracentrifugal lipoprotein patterns (22). It was of interest to observe that heparin also produced a change in the location and character of the lipid zones of the electrophoretogram, as shown by both the Sudan stain and Schultz test. The major lipid band presented a more diffuse appearance and extended further in the anodal direction, so that its peak, which in the pre-heparin specimen had been at, or slightly behind, the β-globulin peak, was nearly at the α_2 globulin. A similar shift toward the anode was sometimes seen in the smaller lipid band associated

‡ This serum specimen was kindly provided by Dr. J. F. Ross, Boston University School of Medicine.

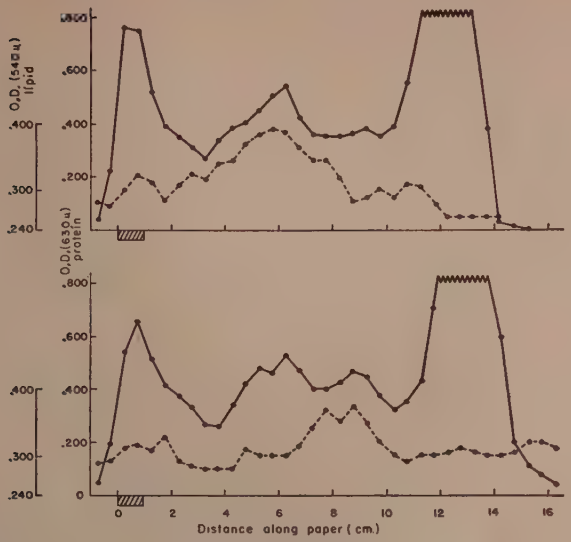

FIG. 3. Electrophoretic patterns of protein and sudanophilic lipid in serum of a male adult before (upper figure), and 35 minutes after (lower figure) the intravenous injection of 8 mg sodium heparin. The subject had ingested 250 cc of heavy cream 3 hr prior to the time the first blood sample was drawn.

with the albumin. The effect was apparent within 15-30 minutes after the intravenous administration of 8 mg sodium heparin, and was observed in the serum of 6 out of 8 fasting subjects and of all 6 of those with alimentary lipemia (Fig. 3). The addition of sodium heparin to serum *in vitro* to a final concentration of 0.1-0.2 mg/cc resulted in changes of a similar nature in the electrophoretic lipid pattern in 6 of the 7 sera so treated, although no clearing of lipemia occurred under these circumstances. In contrast to the marked effect of heparin on the lipid pattern, little change was noted in the protein pattern as demonstrated by bromphenol blue.

Summary. Serum was subjected to electrophoresis on filter paper and the distribution of lipid studied by application to the paper strips of Sudan stains, the Schultz test, and other histochemical procedures. Two principal lipid bands, probably corresponding to β and α lipoproteins, were recognized, the larger being in the β-globulin region, and the smaller in the albumin zone. In some cases lipid apparently associated with little protein was also found at the point of application of the serum. The administration of heparin to human subjects, or its addition to serum, rapidly produced a change in the distribution of sudanophilic and Schultz-reacting lipid.

The author is indebted to Drs. E. B. Astwood and Samuel Proger for helpful suggestions and generous support, and to Dr. C. J. O. R. Morris for advice on the construction of the apparatus.

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Received July 18, 1952. P.S.E.B.M., 1952, v80.

Localization of Proteolytic Activity in Muscle Protein Fractions. (19753)

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In the past most of the studies on muscle have been done in an attempt to learn more about the enzymes involved in the important carbohydrate energy cycle. Consequently, little is known about the proteolytic enzymes in muscle.

The following investigation was undertaken in an attempt to identify some of the proteolytic enzymes and localize them in muscle protein fractions.

Materials and methods. Muscles from the legs of 10 specimens of frog, *Rana pipiens*, were removed and placed in a mixture of ice and water (0 to 2°C) to inhibit bacterial growth and autodigestion. As much connective tissue and as many blood vessels as possible were excluded.

These muscles were then used as a source of muscle protein fractions. Myosin was extracted according to the method of Greenstein and Edsall(1). The procedure of Baranowski(2) was adapted for the extraction of myogen. The procedures outlined by Bate-Smith(3) and Meyer and Weber(4) yielded protein fractions rich in myoalbumin and globulin x, respectively.

A modified method of Anson(5) was employed to obtain some quantitative data concerning the proteolytic enzymes pepsin, trypsin, and cathepsin. Because absolute values could not be ascertained, the amount of proteolytic digestion by increasing quantities of muscle protein during a 10 minute period, was compared with that by standard solutions of pepsin, trypsin, and tyrosine. Standard tyrosine solutions were prepared for comparison with cathepsin because cathepsin is not available commercially.

Results. Very few, small crystals of myogen appeared. They were too small and few in number to separate from the myoalbumin. For that reason and because myogen cannot be extracted from myoalbumin in the native state(3), the myogen and myoalbumin fractions were analyzed together. When preliminary investigations revealed that no proteolytic activity was present in the globulin x fraction, analyses were made only on myosin and myosin-free fractions.

Cathepsin and pepsin were localized in the myosin fraction and trypsin in the myosin-free fraction. No enzyme appeared in both the myosin and myosin-free fractions.

Fig. 1 shows curves resulting from digestion of hemoglobin by increasing quantities of muscle protein fractions and of standard enzyme solutions during a 10 minute period. The curves of digestion by muscle proteins are almost parallel to each other.

Maximum digestion by trypsin in the myosin-free fraction occurred after 6 cc of myosin-

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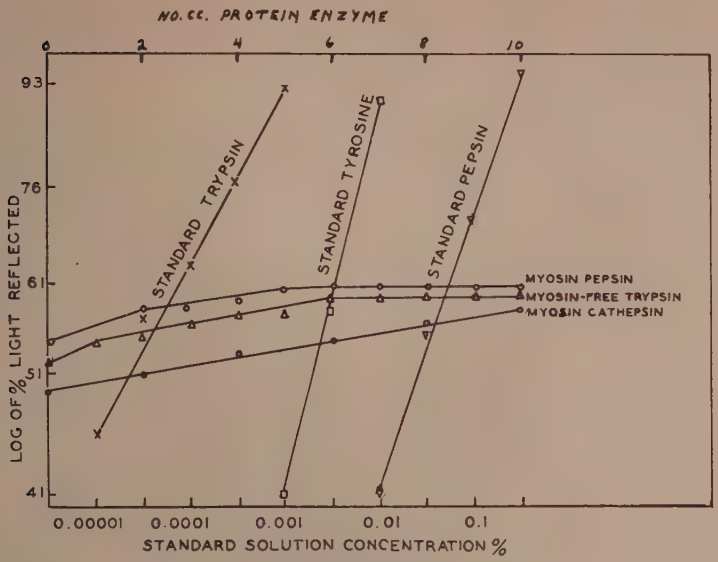

FIG. 1. Amount of digestion by increasing quantities of muscle protein-enzymes during a 10 minute period.

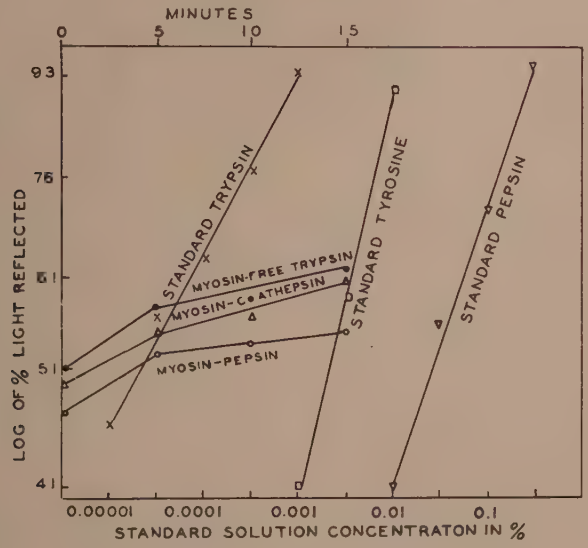

FIG. 2. Amount of digestion by 1 cc of muscle protein-enzymes during varied time intervals.

free solution were added. The total colorimetric change produced (from 52% to 60% light reflected) was comparable to a change produced by increasing the concentration of standard trypsin solution from 0.0004% to 0.0009%. Maximum digestion by pepsin occurred after 5 cc of myosin solution were added. The total colorimetric change (from 55% to 61% light reflected) was comparable to a change produced by increasing the concentration of a standard pepsin solution from 0.05% to 0.075%. There was a straight line

increase in the amount of catheptic digestion by increasing quantities of myosin solution. The overall colorimetric change due to cathepsin digestion (from 40% to 50% light absorbed) was comparable to an increase in concentration of a standard tyrosine solution from 0.0025% to 0.005%. A leveling off phase, indicative of equilibrium, was recorded with myosin-pepsin and myosin-free trypsin solution, but not with myosin-cathepsin solutions.

Curves showing the amount of digestion by proteolytic enzymes in 1 cc of muscle protein solution during different time intervals, are shown in Fig. 2. In all instances there was a rapid increase in the amount of digestion during the first 5 minutes. This was followed by a slower, but steady, increase in the rate of digestion.

Summary. 1. An investigation was made to obtain information regarding the presence and localization of proteolytic enzymes in muscle protein fractions from frog leg muscles. 2. Pepsin and cathepsin were localized in the myosin fraction while trypsin was localized in the myosin-free fraction. No proteolytic activity was displayed by the myoalbumin fraction. 3. The myogen content of the frog muscles is considerably lower than that reported for mammals.

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Received July 18, 1952. P.S.E.B.M., 1952, v80.

Effect of Sodium Monofluoroacetate on Multiplication of Eastern Equine Encephalomyelitis Virus.* (19754)

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Sodium monofluoroacetate has been shown to affect the citrate metabolism of tissues from animals treated with this compound. Potter (1) has found that the citrate level of rat brain after treatment of the animal with this drug was approximately 6 times that of normal. Tricarboxylic acid fractions isolated from tissues of fluoroacetate-treated animals(2) have shown the presence of a fluorine-containing compound ("inhibitor fraction") that would inhibit the oxidation of citrate. Recently it has been found(3) that aconitase activity is inhibited by this "inhibitor fraction". The inhibitory effect is thought to be produced by a fluorine-containing compound other than fluoroacetate and which has been shown to be chromatographically inseparable from the tricarboxylic acid preparations. It has been suggested that the inhibitory substance is fluorocitrate$_2$(4).

Ackermann(5) has found that fluoroacetate will cause an initial depression in the rate of influenza virus production in experimentally infected mouse lung. However, the virus concentration in the treated lung tissue was found to be similar to that of the non-treated lung tissue at a later period in the virus incubation time.

Ainslie(6) has investigated the effect of this compound on the rate of virus production in Lansing poliomyelitis virus infected mice and has noted a significant delay in the virus growth which he was able to relate to an increased survival time of the infected animals treated with fluoroacetate, but the natural course of the infection was not otherwise altered.

Materials and methods. Swiss albino mice

* Aided by a grant from the United States Atomic Energy Commission.

† Post Doctoral Fellow, Institute of International Education.

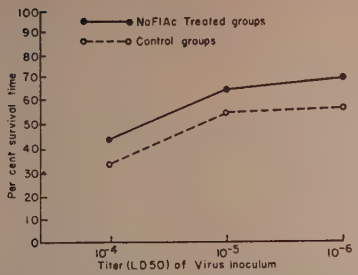

FIG. 1. Effect of sodium monofluoroacetate on the survival time of infected mice.

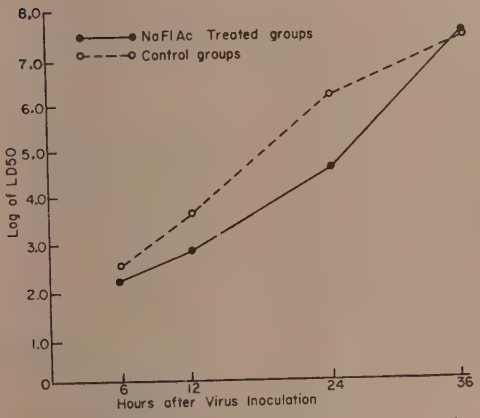

FIG. 2. Effect of sodium monofluoroacetate on the growth curve of virus.

weighing 10 to 15 g (3 to 5 weeks old) were used throughout this work. The mice were matched evenly for weight and age in each experiment.

The fluoroacetate‡ was freshly prepared for intraperitoneal injection by dissolving in isotonic phosphate-NaCl solution (pH 7.4). Mice serving as the "treated" groups were given 5.0 mg/kg of this compound in a volume of 0.15 ml one hour before virus inoculation. This served to eliminate any animals from the experiment that might die from toxic effects of the drug.

Mice were infected by injection of virus intracerebrally, in doses of 0.03 ml, using Eastern equine encephalomyelitis virus.§ Mice were observed for a period of 10 days after infection, although symptoms of infection usually developed within 36 to 72 hours.

Results. Fluoroacetate-treated and normal control animals were separated into groups of 10 animals each and inoculated with varying dilutions of virus (Fig. 1). The per cent survival time was obtained by using a possible ten days of survival for each mouse in the group of 10 animals, thus arriving at the 100% survival time for total survival of the group. There was a consistent increase in the survival time of treated over non-treated control animals.

To evaluate the effect of the fluoroacetate on the survival time of the mice, a larger number of animals were inoculated with a dilute suspension of the virus at "zero time", half of which had been previously treated with fluoroacetate. Five mice from both the treated and control groups were sacrificed at the indicated intervals (Fig. 2) and the brains removed to determine the virus titre (or content) at the given period. The virus concentration was determined by the 10-fold serial dilution method, employing 5 mice for each dilution per virus sample. The LD_{50} dose was calculated by the method of Reed and Muench(7). As can be seen, the fluoroacetate definitely depressed the amount of infective virus in the treated animals during the initial 24-hour period, which has also been shown to be the time of a demonstrable effect of this drug on citrate metabolism in brain tissue(1). However, by the 36 hour period the virus concentrations of the 2 groups are comparable.

It was deemed necessary to determine whether the fluoroacetate could interfere with the amount of virus necessary to establish an infection and, accordingly, titrations of a virus sample were carried out using a) a group of normal mice, and b) a group of fluoroacetate-treated mice. No significant effect of the drug on the susceptibility of the mice relative to virus dosage could be noted (Table I).

The possibility of a direct virucidal action of the drug on the virus was investigated by

‡ The sodium fluoroacetate was kindly supplied by the Monsanto Chemical Co. as a fluorine ion-free compound.

§ This virus was kindly supplied by the Laboratory of the United States Public Health Service, Montgomery, Ala.

TABLE I. *In Vivo* Effect of Sodium Monofluoroacetate on Susceptibility of Mice to Virus Infection.

	NaFlAc-treated group	Control group
Virus sample A (EEE)*	LD_{50} $10^{-6.5}$	LD_{50} $10^{-6.5}$
" " B (EEE)	LD_{50} $10^{-4.5}$	LD_{50} $10^{-4.6}$

* EEE—Eastern equine encephalomyelitis virus.

TABLE II. *In Vitro* Effect of Sodium Monofluoroacetate on Infectivity of Virus (Virucidal Effect).

Virus treated with:	Virus LD_{50}
NaFlAc	$10^{-5.4}$
NaAc	$10^{-5.4}$
Control	$10^{-5.4}$

dividing a virus sample into three parts and diluting them as follows: a) 1:20 to a final concentration of 0.001 M sodium monofluoroacetate, b) 1:20 to a final concentration of 0.001 M sodium acetate, and c) 1:20 with distilled water. These virus dilutions were then incubated at 37°C in a water bath for 1 hour and subsequently titrated in young mice. No significant differences could be noted among the titration results from these three virus samples (Table II.)

Discussion. The results presented indicate that the growth of Eastern equine encephalomyelitis virus is inhibited by the administration of sodium monofluoroacetate in a similar fashion to the inhibition of the viruses of influenza and Lansing poliomyelitis in mice by this drug. The present experiments show that fluoroacetate neither inactivates this virus upon direct contact nor alters the susceptibility of the experimental animal to the virus infection. On the other hand, the experiments do indicate that the chemical prolongs the course of the experimental infection.

The current theory of virus multiplication is that the virus utilizes some of the enzyme systems of the host cell for its reduplication and that this process requires energy. Ackermann(8) claims that for the synthesis of influenza virus, the proper functioning of the Krebs cycle is essential as this cycle seems to offer the energy necessary for the synthesis of some essential component of the virus particle, such as nucleoprotein. As fluoroacetate has been shown to inhibit the oxidation of citrate and consequently to reduce the amount of energy to be gained from the proper functioning of the Kreb's cycle, Ackermann's hypothesis seems to offer a reasonable explanation not only for the reduced synthesis of influenza virus in fluoroacetate-treated tissues, and for that of Lansing poliomyelitis but also for Eastern equine encephalomyelitis virus. Doty and Gerard(9) have shown that fluoroacetate will significantly depress the resting Qo_2 of excised nerve without altering the active Qo_2 or spike height of the stimulated nerve for a period of seven hours. However, the possibility that changes in the cellular metabolism (other than the blocking of energy sources) might produce conditions unsuitable for virus synthesis has not been excluded.

As the rate of infective virus production in the treated tissues was lower than in the control tissues, but not completely inhibited, it is suggested that the reduced production of virus may be due to a) a longer period of virus incubation per infected cell or b) a smaller burst size of virus per infected cell. As the cell population of the brain is by no means homogeneous, it might also be suggested that the virus-sensitive cells may differ in their sensitivities to fluoroacetate, thus limiting the efficiency of the inoculated virus in producing a new generation of infective particles. As the fluoroacetate effect decreases, the efficiency of the virus should increase until the point of saturation of susceptible cells is approached(10).

Summary. The reduplication of Eastern equine encephalomyelitis virus in mouse brain is delayed in mice previously treated with sodium monofluoroacetate. This chemical seems to prolong the survival time of the infected animals, although it does not otherwise alter the course of infection. In high concentration, the chemical has no direct effect *in vitro* on the infectivity of the virus, nor does it show an *in vivo* effect on the susceptibility of the host to the infection with this virus.

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Received July 21, 1952. P.S.E.B.M., 1952, v80.

Inhibition of Aminotripeptidase.* (19755)

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An aminoexotripeptidase which hydrolyzes one peptide bond of a tripeptide such as l-leucylglycylglycine and diglycylglycine (GGG) has been demonstrated in a number of tissues by Ellis and Fruton(1). This enzyme, referred to by these authors as tripeptidase, was inhibited by a number of metal ions and by relatively large concentrations of cysteine, but not appreciably by cyanide, sulfide or citrate ions. Although lymphoid cells are said to be especially rich in tripeptidase(2), it is distributed in other cell types (3), and has been found in several microorganisms(4). Stern and co-workers(5), have shown that human white blood cells have a high tripeptidase content, and that the activity of leucocytes was about 40 times greater than that of erythrocytes. The tripeptidase activity of serum is increased in certain pathological conditions such as bone fracture(6), burns(7) and a number of diseases(8). It has been observed in this laboratory that a tripeptidase is present in synovial effusions of a number of arthritic diseases, and is especially rich in the synovial fluid of patients with rheumatoid arthritis(9).

We have observed, in the present investigation, that the activity of tripeptidase from calf thymus, rabbit skeletal muscle and human synovial fluid, with respect to the hydrolysis of GGG, is inhibited by a group of compounds possessing anti-histamine, local anesthetic and anti-cholinesterase activity in concentrations as low as 0.0001 M.

Methods. Peptidase activity was determined using the titrimetric method of Grassman and Heyde(10). The reaction mixture contained 0.5 or 1.0 ml of the enzyme solution, 0.5 ml of 0.25 M GGG, previously neutralized to pH 7.8, 0.1 ml of inhibitor solution neutralized to pH 7.8 just prior to use, and sufficient 0.02 M veronal buffer at pH 7.8 to bring the total volume to 2.5 ml. The reaction was carried out in stoppered flasks and in a shaking water bath at 37.5°C. In general, reaction was allowed to proceed to 60% hydrolysis of one peptide bond, and 4 or 5 analyses in duplicate were obtained by titration of 0.2 ml samples, removed from the incubation mixture, with 0.015 N KOH in 90% ethanol. The hydrolysis of GGG was zero order to the extent of approximately 60% splitting of one peptide bond. Results are expressed as per cent hydrolysis per hour.

Calf thymus peptidase extracts were prepared according to Fruton(2), and rabbit skeletal muscle extracts according to Smith (11). The extracts obtained were frozen and thawed twice to remove extraneous protein without diminution in total activity. When inhibitor compounds were added, the reaction was followed for a variable time given below, dependent on the extent of inhibition. In the case of benadryl and procaine, the hydrolysis curves were not linear because of decomposi-

* This investigation has been supported by a grant from the Masonic Foundation for Medical Research and Human Welfare.

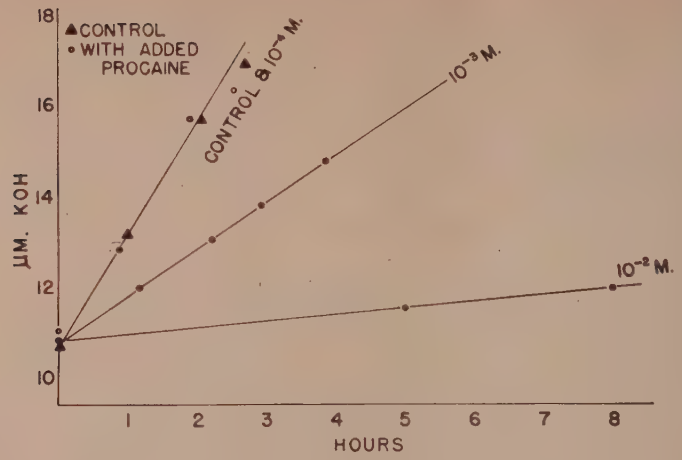

FIG. 1. Effect of procaine on hydrolysis of GGG by calf thymus extract (N = .0714 mg/ml); μM GGG = 1250; pH = 7.8, veronal buffer. Curve at 10^{-2} M procaine concentration is corrected for titration of procaine which represented 2.1 μM, as determined on solutions at zero time.

tion of these substances in the muscle extract during the incubation(12,13). As an approximation, in the case of these two substances, the rate of hydrolysis was obtained by drawing a smooth curve and calculating the rate from the initial 60-minute portion of the curve.

Synovial fluid was obtained by aspiration of knee joints of patients with rheumatoid arthritis using ethyl chloride anaesthesia. The fluid was promptly centrifuged at 3000 rpm for 20 minutes. When stored in the refrigerator, tripeptidase activity of synovial fluid did not decrease over a period of two months. The inhibiting effect of the following substances was studied: Procaine hydrochloride, pronestyl (procaine amide hydrochloride), benadryl (β-dimethylaminoethyl benzhydryl ether hydrochloride), pyribenzamine (N′-pyridyl-N′-benzyl-N-dimethylethylenediamine hydrochloride), cocaine hydrochloride, eserine salicylate, sodium salicylate, diisopropylfluorophosphate (DFP), and tetraethylpyrophosphate (TEPP).

Results. The hydrolysis of GGG by calf thymus extract in the presence of procaine is shown in Fig. 1. Marked inhibition was observed in the presence of 0.001 M procaine. Fig. 2 presents the results of a similar experiment using the synovial fluid obtained from a patient with rheumatoid arthritis. It is noted that in contrast to the constant rates observed with the thymus enzyme extract, increasing rates of hydrolysis were found with

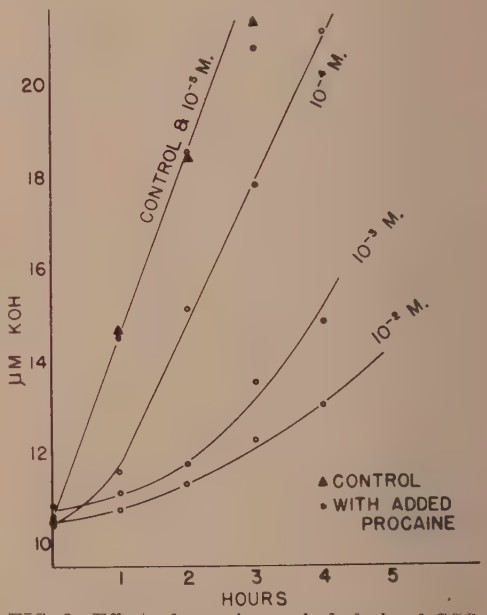

FIG. 2. Effect of procaine on hydrolysis of GGG by synovial fluid from a patient with rheumatoid arthritis (N = 42 mg/ml). Curve at 10^{-2} M procaine concentration is corrected for titration of procaine which represented 2 μM as determined on solutions at zero time.

TABLE I. Inhibition of Rabbit Skeletal Muscle Tripeptidase. Concentration of GGG in incubation mixture .05 M; nitrogen of tripeptidase test solution 2.79 mg/ml; final concentration of all substances listed below was .001 M.

Added substance	% of original tripeptidase activity
None	100
Pyribenzamine	8.7
Benadryl	25.8*
Procaine	30.4*
Eserine	31
Cocaine	41
Pronestyl	57.7
Sodium salicylate	100
Diisopropylfluorophosphate	100
Tetraethylpyrophosphate	100

* Calculated from initial 60 min period.

synovial fluid, suggesting the simultaneous hydrolysis of procaine in synovial fluid, probably by an independent esterase reaction(14).

Pyribenzamine, benadryl, procaine, eserine, cocaine, and pronestyl were found to be effective inhibitors of rabbit muscle tripeptidase as shown in Table I. DFP and TEPP, powerful inhibitors of cholinesterase, did not inhibit tripeptidase when present in relatively high concentration.

Discussion. Ellis and Fruton(1) have prepared highly purified tripeptidase from calf thymus. This enzyme demonstrated marked specificity towards optically active tripeptides, and showed little or no activity with respect to dipeptides, tetrapeptides, tripeptides containing β-amino acids, and substrates split by endopeptidases. In view of this, they concluded that calf thymus tripeptidase is specifically adapted to act on peptide chains of a particular chain length characteristic of tripeptides composed of α-amino acid residues.

It is shown in Fig. 3 that the structures of the substances found to be inhibitors approximate the dimensions of the tripeptide substrate. All are tertiary amines and, like GGG, have a positively charged onium group at the pH of the reaction mixture, 7.8. The dense lines trace the longest chain length from the onium nitrogen atom, which for all substances is either 8 or 9 carbon atoms. It would appear,

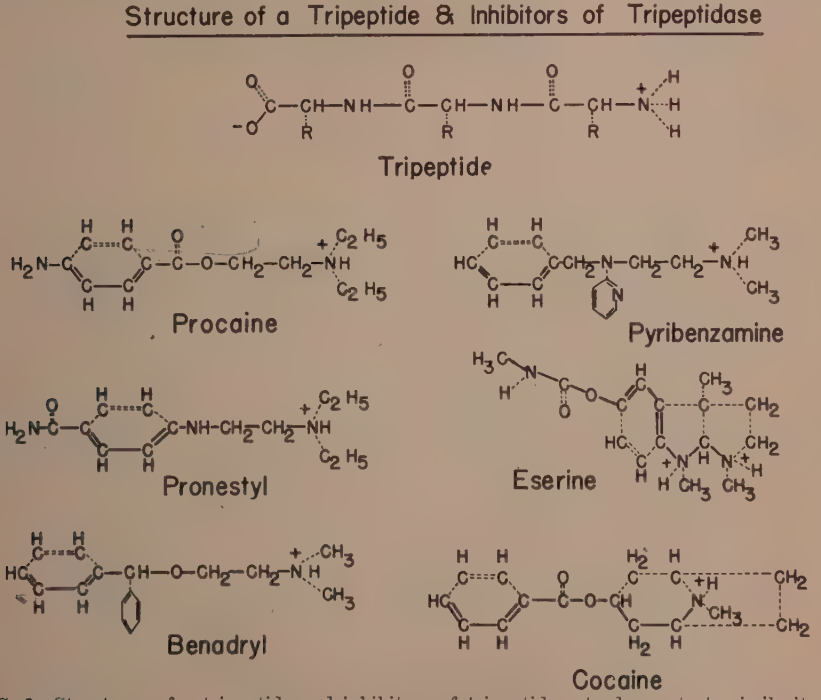

FIG. 3. Structures of a tripeptide and inhibitors of tripeptidase to demonstrate similarity in chain length and presence of onium groups.

therefore, that the inhibition observed is related to similarity of inhibitor to substrate, both as to structure and charge.

It has been shown that both true and pseudocholinesterase are inhibited by DFP (15). Jansen and Balls(16) suggest that the mode of inhibition by DFP of all esterases may be similar to the observed inhibition by this agent of both the esterolytic and proteolytic activity of β and γ chymotrypsin and of trypsin. Since DFP did not inhibit the tripeptidase of rabbit skeletal muscle (Table I) and in view of the apparent inability of the purified calf thymus tripeptidase to hydrolyze procaine, as indicated by the constant slope of the hydrolysis rate of GGG in the presence of procaine, it would appear that tripeptidase does not have esterolytic properties.

The observation that the group of compounds studied are inhibitors of aminoexotripeptidase, which is widely distributed in a variety of tissues, raises the question of the relationship between peptidase inhibition and the pharmacological action of these substances. Pyribenzamine, benadryl, procaine, cocaine, and eserine are local anaesthetic agents, and block nerve conduction(17). DFP, which did not inhibit tripeptidase in the present investigation, does not block nerve conduction in concentrations which completely inactivate nerve cholinesterase(18). The possibility arises, therefore, that peptidase inhibition may play a role in local anaesthetic activity. Whether the local effects of anihistaminic substances such as pyribenzamine and benadryl in inflammatory skin disease, as contact dermatitis and eczema(19), are related to peptidase inhibition cannot at present be determined.

Summary. A group of compounds known to possess antihistamine, local anaesthetic, and anti-cholinesterase activities were observed to inhibit aminoexotripeptidase from several sources in concentrations as low as 0.0001 M. The inhibiting substances were pyribenzamine, benadryl, procaine, pronestyl, cocaine and eserine; diisopropylfluorophosphate and tetraethylpyrophosphate did not inhibit. The mechanism of the observed inhibition and its possible physiological significance is discussed.

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Received July 21, 1952. P.S.E.B.M., 1952, v80.

Effect of Water and Electrolyte Restriction on Pathologic Changes in Kidney of Jaundiced Rats. (19756)

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The occurrence of reduced renal function (and renal injury) in the presence of hepatic insufficiency or jaundice has long been recognized. This present study was made in order 1) to investigate the early pathologic changes in the kidney of the rat following the production of jaundice by occlusion of the bile duct and 2) to determine the influence of restricted intake of water and electrolytes on such changes. The lesions observed in the kidney of the rats studied were comparable to those associated with jaundice in man and in other experimental animals as described by other investigators(1-4). In general, restriction of water and electrolytes appeared to exaggerate the severity of the lesions.

Methods and results. The bile duct was occluded in albino rats weighing 100 to 250 g. General anesthesia was employed for all surgical procedures. After the desired number of days, during which jaundice developed, the rats were killed, and the kidneys were removed and placed immediately in a 10% solution of formalin for fixation. Sections were stained with hematoxylin and eosin for microscopic study.

Fourteen rats were killed and the kidneys were removed 2 to 11 days after complete obstruction of the bile duct. In 4 of these rats tubular damage was observed. The kidneys of 10 other rats were removed 4 to 11 days after complete obstruction of the common bile duct and, in addition, after the concomitant restriction of water for 4 to 7 days. In 8 of these 10 animals tubular damage was present. However, the animals in this second group lost considerable weight and were severely dehydrated.

Grossly, the kidneys of both of these groups of rats were diffusely bile stained. The surfaces of some of the kidneys were mottled. The predominant microscopic change was vacuolation in the cytoplasm of the tubular cells, especially the proximal convoluted tubules (Fig. 1 and 2). In 2 rats of the series in which intake of water was restricted the tubular changes were severe and considerable necrosis was present. A few scattered casts were seen

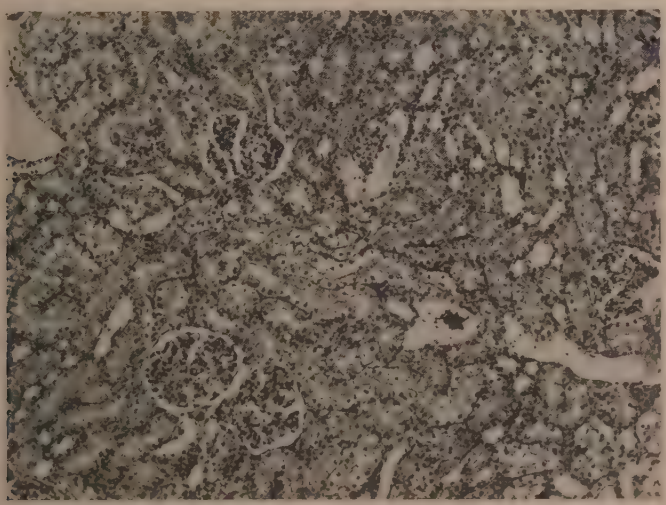

FIG. 1. Vacuolar degeneration and necrosis of tubular cells of the kidney of the rat associated with jaundice and severe restriction of water (hematoxylin and eosin; × 115).

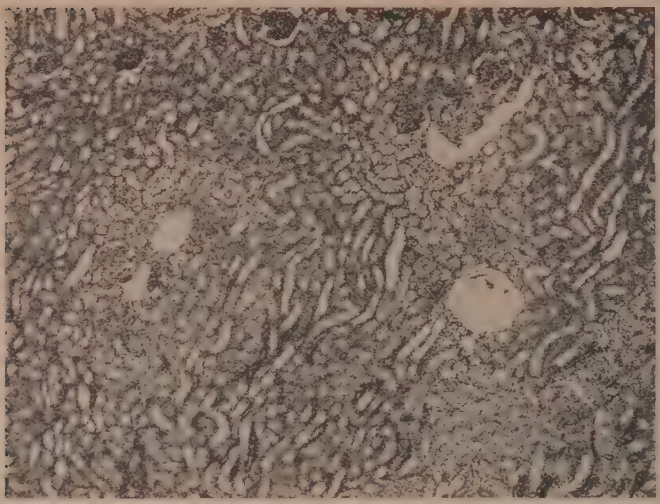

FIG. 2. Focal areas of vacuolar degeneration of tubular cells of the kidney of the rat associated with jaundice and severe restriction of water (hematoxylin and eosin; × 60).

in the tubules. A few casts resembled pigmented casts and were stained orange-red. Scattered hyaline and granular bile-stained casts were present. Usually casts were a relatively inconspicuous histologic feature but a number of casts may have been lost during the preparation of the sections. A few scattered dilated tubules were also present. The renal changes did not appear sufficiently severe to disturb renal function seriously. The tubular changes usually appeared most marked in the cortex and were usually restricted to discrete areas of varying size. The changes were not predominant in any one segment of the nephron. The glomeruli appeared normal. In 6 of 8 rats in which the effects on the kidney of severely restricted intake of water alone were studied, small focal areas of vacuolar degeneration of tubular cells were present.

In a series of 8 additional rats which had been receiving distilled water and a diet free of inorganic salts for 4 to 5 weeks, the bile duct was occluded. These rats either failed to gain weight or lost weight while on the diet but drank adequate amounts of water *ad lib.* The kidneys of all of these rats contained varying degrees of gross and microscopic changes similar to those just described. However, in the kidneys of 2 of these animals bile-stained casts were prominent and the microscopic changes resembled those of hemoglobinuric nephrosis (Fig. 3). The casts were located for the most part in the distal tubules and especially in collecting tubules and were most numerous in the corticomedullary region. Granular bile-stained casts were most numerous, but hyaline casts which stained bright red and casts of degenerating tubular cells were present also. Tubular dilatation, lymphocytic infiltration, tubular degeneration and necrosis, mitotic figures, multinucleated giant cells in tubular lumina were all present. Grossly, the cut surface of the kidneys of one of these rats showed a deep yellow band at the corticomedullary region.

In other experiments on the effect of prolonged intake of food and water free of inorganic salts on the kidney of the rat we have frequently observed tubular degeneration of varying severity and extent in the absence of jaundice. Therefore, in the experiments in which jaundice was produced after prolonged restriction of salt, the renal changes that followed the occlusion of the bile duct were probably superimposed on and possibly dependent on those associated with the restriction of salt.

Comment. This study indicates that degenerative changes in the renal tubules may be associated with jaundice produced by occlu-

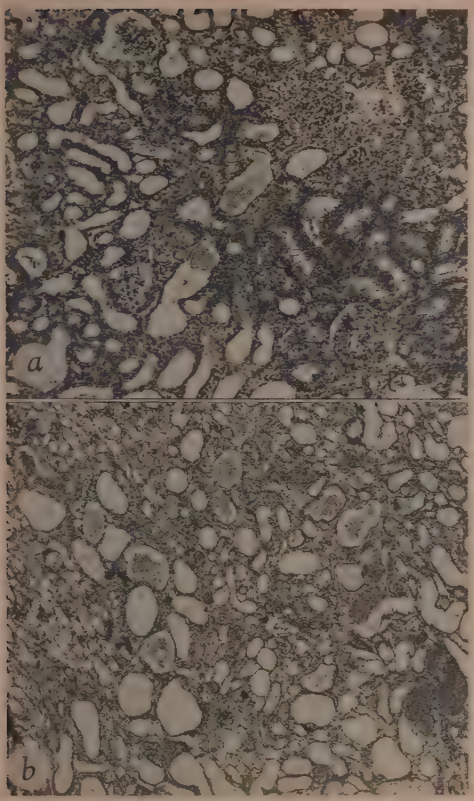

FIG. 3. Bile-stained and hyaline casts, tubular degeneration and necrosis plus tubular dilatation in the kidney of the rat following production of jaundice and restriction of intake of inorganic salts (hematoxylin and eosin: a, × 75; b, × 50).

sion of the bile duct. However, the lesions do not appear severe or extensive. Furthermore, other factors such as dehydration from depletion of electrolytes or water or both appear to intensify the severity of the lesions. Depletion of water had to be severe, however, to influence the microscopic findings during the period of observation. Wartman and associates(3) reported that obstruction of one renal artery increased the incidence and severity of renal changes after occlusion of the bile duct in the rabbit. A number of investigators have reported vacuolation and necrosis of the cytoplasm of renal tubular cells in water and electrolyte deficiency in experimental animals(5-10). These changes are different from those of osmotic nephrosis. We too observed that areas of tubular degeneration and necrosis occur in kidneys of rats after exclusion of inorganic salts from the diet over a long period. The renal lesions appear more severe and extensive when jaundice and electrolyte and water depletion are combined than when each condition is present separately.

Whether ischemia plays a role in the production of renal lesions as seen in this study is unknown. The microscopic features of the lesions could not be differentiated from the vacuolar degeneration and tubular necrosis we have observed in renal tubules resulting from ischemia produced by temporary occlusion of the renal artery or severe acute hemorrhage (11). In 5 jaundiced rats of the present study India ink was injected intravenously prior to removal of the kidneys. The ink was uniformly distributed throughout the kidneys, including the areas in which microscopic changes were observed.

In general, 2 types of renal lesions were seen associated with jaundice in rats in this study. The first and most common consisted essentially of tubular vacuolar degeneration, varying greatly in extent and severity, and associated with a few casts and dilated tubules. In more severe lesions, necrosis of tubules occurred. The second but relatively infrequent lesion resembled that of hemoglobinuric nephrosis with the exception that the casts appeared to be bile casts. This latter type of renal lesion may be the result of the superimposition of the effects of jaundice on kidneys containing recent degenerative tubular changes due to other causes. Of course, a mixture of the 2 patterns of lesions listed may occur. Ayer(2) found that in infants dying with jaundice from congenital atresia of bile ducts the renal lesions were similar to hemoglobinuric nephrosis. Thompson and associates(1) emphasized that the renal damage present in cholemic nephrosis was intensified when a preexisting lesion was present. Lalich(12) found that injections of homologous hemoglobin into rabbits previously dehydrated by water restriction resulted in hemoglobinuric nephrosis, if the dehydration was sufficiently prolonged. Thus, the pathogenesis of the renal lesions in the 2 entities, bile nephrosis and hemoglobinuric nephrosis, may be similar.

Summary and conclusions. Severe deple-

tion of water or electrolytes or both appears to increase the incidence and severity of renal lesions associated with jaundice produced by occlusion of the bile duct in rats. The renal lesions under these conditions may vary greatly in extent and severity but usually do not appear sufficient to produce either significant or fatal renal insufficiency. They usually consist of vacuolation of the tubules, especially proximal convoluted tubules, with a few casts and dilated tubules. Necrosis of tubules occasionally occurs. The renal lesions in this study infrequently closely resembled those of hemoglobinuric nephrosis. The casts, however, appeared to be mostly bile-stained casts. Rarely the lesion was extensive enough to cause severe renal damage.

The renal lesions seen in bile nephrosis then may be due in part to or may be superimposed on those due to severe electrolyte and water depletion or other causes of renal tubular degeneration.

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Received July 21, 1952. P.S.E.B.M., 1952, v80.

Concentrations of Nineteen Amino Acids in Plasma and Urine of Fasting Normal Males.* (19757)

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The data on the concentrations of individual amino acids in the plasma and urine of normal humans are somewhat limited. Furthermore, the findings which are available are scattered, and, until recently, have been confined to reports concerned with one or two of those amino acids which could be determined chemically. Specific quantitative chemical methods have been applied only to the determination of glutamine(1-3), glutamic acid (4,5), glycine(6), alanine(7), arginine(8), and tyrosine(9), and normal levels of these substances have been reported for human plasma and urine. However, since the introduction of microbiological and chromatographic technics, it has become possible to assay biological fluids for almost all of the amino acids of physiological importance. Therefore, it is now feasible to study variations in plasma and urine concentrations of at least 19 individual amino acids both in health and in disease. Some studies of this nature have been reported. Ackermann *et al.* (10,11) have studied plasma levels of 9 amino acids in human subjects of various age groups. Kirsner *et al.*(12,13) and Steele *et al.*(14) have applied microbiological methods to studies of the effect of variations in protein intake on levels of amino acids in human plasma and urine. Hier and Bergeim(15) reported plasma levels of 10 amino acids determined microbiologically in normal human beings. Harper *et al.* have reported on the normal levels of

* The opinions expressed in this paper are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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methionine(16) and glutamine and glutamic acid(17), also determined microbiologically. It is the purpose of this paper to report the simultaneous determination of the plasma and urine levels of 19 amino acids as observed in fasting, healthy, young males.

Procedure. The subjects chosen for this study were young males on active duty in the U. S. Navy. They were, for the most part, staff members of the U. S. Naval Hospital, Oakland, Calif., ranging in age from 18-30 years. Food was withheld after the evening meal and at 7:00 a.m. the following day the bladder was emptied. All urine excreted in the 2-hour period between 7:00 and 9:00 a.m., was collected for amino acid assay and a sample of venous blood was then drawn for determination of the plasma amino acid levels following this 12- to 13-hour fast. Heparin was used as anticoagulant.

Diet is known to have a marked influence on the excretion of a number of amino acids (12-14). For this reason the urinary amino acids were determined on specimens voided after a fast of 12-13 hours. It is considered that this technic minimized individual variations attributable to the previous diet.

Microbiological assays for 19 amino acids were carried out on the urine specimens and on the filtrates of plasma deproteinized by treatment with 5% acetic acid at 100°C. Arginine and threonine were determined using the basal medium described by Henderson and Snell(18) with *Streptococcus faecalis* (ATC, 8043) as test organism. In the assay of the other amino acids the medium described by Steele *et al.*(19) was used. One-half milliliter of basal medium was added to each assay tube with supplements to a total volume of 1 ml except in the case of glutamic acid and glutamine, where double these quantities was used. The acid produced after 72 hours incubation was measured by titration with N/100 NaOH, or N/50 NaOH when a total volume of 2 ml was employed (glutamine and glutamic acid). *Leuconostoc mesenteroides* P-60 (ATC, 8042) was the test organism for the assay of aspartic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, and tyrosine; *Lactobacillus buchneri* (ATC, 4005) for cysteine-cystine; *Leuconostoc citrovorum* (ATC, 8081) for alanine; and *Lactobacillus arabinosus* 17-5 (ATC, 8014) for valine, glutamic acid and glutamine. The method of Harper(17) was utilized for the assay of glutamic acid and glutamine; that of Hutchin *et al.*(20) for cysteine-cystine. In the assay for glutamine and for cysteine-cystine the samples for assay and the standard solutions were sterilized by Seitz filtration and added aseptically to the tubes containing the basal medium which had been previously heat-sterilized and cooled.

It is to be noted that the amino acid levels observed represent only free amino acids, since no attempt was made to derive additional amino acids by hydrolytic procedures.

Results and discussion. The mean plasma levels of the 19 amino acids studied are recorded in Table I. The number of subjects, the ranges of plasma concentration, and the standard deviations are also shown in this table. For purposes of comparison the results of other observers have been supplied. With respect to most of the amino acids the data from our study agree well with the results of the authors previously cited. The only noteworthy exceptions were in the cases of glycine, histidine, phenylalanine, and tryptophan (mean varied by more than one standard deviation from that of previous authors). When the large number of physiological processes in which glycine is implicated is considered, the observed variability in the plasma levels of this particular amino acid is not surprising.

Marked variations in the amount of each amino acid excreted during the 2-hour test period were noted. However, with respect to the absolute quantities found in the urine, the amino acids could be conveniently divided into 4 groups. Glycine (3-36 mg/hr, mean 11.7 $\pm$ 9.45) and histidine (2.7-23.0 mg/hr, mean 7.75 $\pm$ 1.33) comprised the first group. These 2 were uniformly excreted in the largest amounts. Glutamine (1.6-4.6 mg/hr, mean 2.87 $\pm$ 0.96), and cystine (0.4-4.3 mg/hr, mean 2.30 $\pm$ 1.22) were intermediate. Leucine and isoleucine, valine, aspartic and glutamic acids, and methionine were excreted in small amounts (maximum, less than 1 mg/hr), while the remaining 9 amino acids were found

TABLE I. Plasma Levels of 19 Amino Acids in Fasting Normal Male Subjects.

Amino acid	No. of subjects	Observed plasma levels (mg/100 ml)			Reported plasma levels (mg/100 ml)			
		Range	Mean	S.D.	Range	Mean	S.D.	Ref.
Glutamine	14	4.6 –10.6	7.51	1.63	5 –12	—	—	1
					5 –12	—	—	2
					2.7 – 7.8	—	—	4
					—	8.3	1.5	5
					6.1 –13	—	—	17
Alanine	17	2.4 – 7.6	3.96	1.47	—	3.97	.7	7
					3.4 – 4.9	—	—	14
					3.2 – 6.4	—	—	21
Lysine	17	2.3 – 5.8	3.68	1.15	1.8 – 4	2.85	.57	11
					2.1 – 3.1	—	—	13
					3.7 – 4.2	—	—	14
					—	2.95	.42	15
Valine	16	2.5 – 4.2	3.23	.53	—	2.86	.42	11
					2 – 3.4	—	—	13
					2.8 – 3.8	—	—	14
					—	2.83	.34	15
Cysteine-cystine	13	1.8 – 5	3.02	1.16	—	—	—	—
Glycine	17	.8 – 5.4	2.91	1.36	—	1.77	.26	6
					—	1.26	.23	11
					.6 – 1.6	—	—	14
					1.5 – 2	—	—	21
Proline	17	1.5 – 5.7	2.61	1.26	2.6 – 3	—	—	14
Leucine	17	1 – 5.2	2.48	.90	—	1.89	.29	11
					2 – 2.8	—	—	13
					1.7 – 3.3	—	—	14
					—	1.91	.34	15
Arginine	16	1.2 – 3	2.26	.47	2 – 2.4	—	—	8
					1.5 – 1.9	—	—	13
					1.6 – 3.6	—	—	14
					—	2.34	.62	15
Histidine	17	1 – 3.8	2.11	.61	—	1.34	.17	11
					1.7 – 2.5	—	—	13
					2.7 – 3.9	—	—	14
					—	2.02	.45	15
Threonine	17	.9 – 3.6	2.06	.69	—	1.88	.42	11
					1.7 – 2.5	—	—	13
					2.7 – 3.9	—	—	14
					—	2.02	.45	15
Isoleucine	17	1.2 – 4.2	2	.79	—	1.33	.19	11
					1.6 – 2.1	—	—	13
					1.6 – 2.3	—	—	14
					—	1.60	.31	15
Phenylalanine	17	1.1 – 4	1.99	.82	1.5 – 2.4	—	—	14
					—	1.38	.32	15
Tryptophan	17	.9 – 3	1.74	.51	.8 – 1.5	1.18	.20	11
					1 – 2.3	—	—	14
					—	1.08	.21	15
Serine	17	.3 – 2	1.39	.44	—	—	—	—
Tyrosine	17	.9 – 2.4	1.32	.37	1 – 1.8	1.4	—	9
					—	1.38	.22	11
					.9 – 1.5	—	—	14
					—	1.48	.37	15
Glutamic acid	17	.0 – 1.3	.89	.39	1.3 – 5.9	—	—	4
					—	1.1	.5	5
					.0 – 1.4	—	—	17
Methionine	18	.25– 1	.57	.27	.4 – .7	—	—	13
					.8 – 1.1	—	—	14
					.5 – 1.5	.85	—	16
Aspartic acid	17	.0 – 1.2	.33	.37	.6 – .9	—	—	14

in amounts ranging from 1.0-3.4 mg/hr.

The considerable variability in the normal excretion of any one amino acid which we have noted has also been observed by other workers(12-14). It illustrates the difficulties likely to be encountered in interpreting or ascribing significance to amino acid levels in urine.

Summary. The plasma and urine levels of 19 free amino acids as determined microbiologically in fasting, healthy, young males are reported.

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Received July 22, 1952. P.S.E.B.M., 1952, v80.

An Anthrone Procedure for Determination of Inulin in Biological Fluids. (19758)

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The use of inulin for extracellular space estimation, as well as clearance measurements, has increased the need for a simpler and more accurate method for the determination of this substance in biological fluids. At present, resorcinol(1,2) and diphenylamine(3) are most frequently used for this determination. The former has a greater specificity for fructose, but is somewhat less reproducible. While greater accuracy may be obtained with diphenylamine, this method is more tedious.

Recently, Morris(4) has described the quantitative determination of carbohydrates with Dreywood's anthrone reagent. The present report describes the application of this method to the measurement of inulin in plasma and urine. A modification of Dreywood's anthrone reagent makes possible the measurement of fructose under controlled temperature conditions and gives highly reproducible results. Glucose and other alkali-labile chromogens are destroyed by sodium hydroxide digestion(5) so that inulin can be determined in the presence of high concentrations of these materials. It has been found that commercial inulin contains a considerable amount of alkali-labile carbohydrate material that can be completely removed by further purification (6).

Methods. Reagent: To prepare one liter of anthrone reagent, 500 ml of concentrated sulfuric acid are added to 250 ml of water, and the mixture is cooled to room temperature. Four grams of anthrone (Eastman Organic

Chemicals) are dissolved in 250 ml of concentrated sulfuric acid and the solution is added to the diluted acid. Over-heating destroys the reagent and must be avoided. The reagent is stable up to 4 months if kept in brown bottles at room temperature. Color Development: Ten ml of anthrone reagent are added to two ml of standard or unknown solutions containing from 15 to 45 μg of inulin (William R. Warner Co. inulin ampules, Lot No. 016091, are used throughout unless otherwise stated). During the addition of the acid solution, the tube is shaken in a cool water bath to minimize heat development. After thorough mixing the samples are heated in a constant temperature water bath at 75°C ($\pm$0.5°) for 5 minutes. The tubes are cooled in tap water, and after 15 minutes the color is read at 630 mμ in a Coleman Junior Spectrophotometer.

Plasma: Plasma proteins are precipitated by Somogyi's method(7).* For plasma inulin concentrations of 15 to 45 mg %, 13 ml of water, one ml of 10% $ZnSO_4 \cdot 7H_2O$ and one ml of 0.5 N NaOH are added to one ml of plasma. The mixture is placed on an automatic shaker for 30 minutes. By this means 98% of added inulin can be recovered from plasma whereas only 92% is recoverable after brief manual shaking. One ml of 4 N NaOH is added to a 4 ml aliquot of the plasma filtrate making a final dilution of 1:20. Each tube is covered with a glass marble and placed in boiling water for 15 minutes. The tubes are cooled, and duplicate 2 ml aliquots are used for the development of color.

Urine: Protein-free urine is diluted so that inulin concentrations approximate that of the plasma filtrate: 4 ml aliquots are digested with sodium hydroxide and analyzed in the same manner as plasma filtrates.

Results. I. Conditions for optimal color development: The absorption maximum was found to be 630 mμ in both the Coleman Junior and Beckman Model B Spectrophotometers. Fig. 1 illustrates the effect of temperature on the color developed after 5 minutes

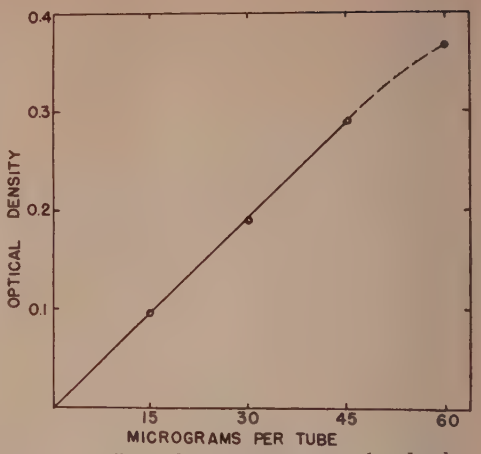

FIG. 1. Effect of temperature on color development. All tubes contained 60 μg of undigested inulin and were heated for 5 min.

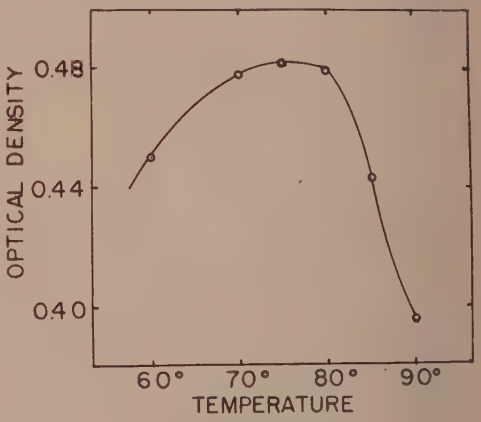

FIG. 2. Inulin standard curve.

heating between 60 and 90°C.† Maximal color is obtained at 75°C, but as seen in Fig. 1, small variations in bath temperature between 70 and 80°C do not greatly influence color development. The color is stable at room temperature for 4 hours.

II. Sensitivity and reproducibility. Fig. 2 shows the standard curve obtained with 15, 30, 45 and 60 μg of inulin per tube (equivalent to 15 to 60 mg % in plasma). This curve obeys the Beer-Lambert law up to 45 μg per tube. Curves prepared from determinations

* Cadmium sulfate and trichloroacetic acid give incomplete recoveries, and sodium tungstate is found to give a color with anthrone.

† Heating for longer than 5 min. serves only to reduce the color; *e.g.*, heating for 10 min. yields only 97% of the color developed by 5 min. heating.

TABLE I. Reproducibility of Optical Densities with Standard Inulin Solutions.

Concentration, μg/tube	No. of determinations	Optical densities	Range O.D.	Stand. dev. O.D.	Stand. dev., μg
15	14	.097	± .002 .003	± .002	± .30
30	23	.189	± .002 .007	± .002	± .38
45	10	.289	± .001 .004	± .002	± .31
60	14	.367	± .009 .007	± .005	± .82

TABLE II. Recoveries of Inulin (30 mg %) Added to Plasma and Urine and Urinary Recoveries of Infused Inulin.*

	Plasma		Urine		% urinary recovery of infused inulin
	mg % recovered	% recovery	mg % recovered	% recovered	
Avg	29.3	97.7	29.9	99.7	99.6†
Range	+.8–1.2	+2.5–4	+1–1.3	+3.1–4.5	+2.9–1.6

* 10 experiments were performed.
† One of these recoveries represented a recovery of repurified inulin (see text).

with fresh standards over a period of 2 months show a high degree of reproducibility (Table I). Between 15 and 45 μg per tube the results give a standard deviation of only 0.3 to 0.4 μg or 0.7 to 2.0%.

III. Sodium hydroxide digestion. Sodium hydroxide digestion effectively destroys interfering chromogens in plasma and diluted urine. The optical densities of plasma and urine blanks were essentially zero except for one of 14 plasma blanks in which the optical density was 0.005 (equivalent to 0.8 mg % inulin). A comparison of the effects of added glucose on blank chromogen values determined by Roe's(1) resorcinol method and our method was made. The blank values (expressed as inulin equivalent), obtained by Roe's method from the addition of 250, 500 and 1,000 mg % glucose, ranged from 2.4 to 3.0, 4.5 to 6.1, and 8.3 to 11.3 mg %, respectively. However, using sodium hydroxide digestion and the anthrone reagent, the values only ranged from 0.0 to 0.4, 0.1 to 1.0, and 1.3 to 2.5 mg % respectively.

Sodium hydroxide digestion destroys about 25% of the chromogenic material in both ampuled and dry Warner's inulin and about 10% of another dry preparation (Fisher Scientific Co. C.P.). This destruction is complete in 15 minutes and there is no further loss after 25 minutes digestion. The loss also appears when resorcinol is used to measure chromogen. Treatment of Fisher's inulin by sodium hydroxide digestion followed by repeated recrystallization yields an apparently pure material that is alkali-stable and gives the same color per unit weight as fructose (Fisher Scientific Co. C.P.). This repurified inulin was tested by infusion in a normal dog and gives the same recovery and volume of distribution as the material in ampules.

IV. Recoveries from plasma and urine. The recovery of inulin added to plasma and urine is shown in Table II. The average of 10 recoveries from plasma is 97.7% with a standard deviation of ±2.53% and from urine is 99.7% ± 2.0. Using the recovery method (8) for inulin space determinations in normal dogs we obtained urinary recoveries of 99.6% (standard deviation of ±1.5%) of the infused inulin when urine collections were continued for 5 hours after infusion.

Discussion. The anthrone method has the advantage that the reagent is stable, simple to prepare, and easier to handle than resorcinol or diphenylamine, a highly accurate constant temperature bath is not required, and the final color is stable. The method appears to have as great a sensitivity and somewhat greater reproducibility than other procedures. Sodium

hydroxide digestion appears to be more effective with anthrone than with diphenylamine in that the problem of interference from glucose and other blank chromogens is solved without the addition of glucose suggested by Little(5).

Cotlove(9) has found that commercial inulin contains 3 fractions: about 1% is a reducing material which diffuses like fructose, 15 to 40% is non-reducing but alkali-labile and diffuses like a much larger molecule, while the remainder is non-reducing and alkali-stable and diffuses even slower. He could detect no difference in the renal clearance and distribution in muscle between the total non-reducing and the alkali-stable fractions. Weil(6) has reported that 34% of commercial inulin is alkali-labile and feels that purified material by recrystallization after alkali digestion is more suitable for analysis of inulin in muscle. We have compared inulin and creatinine clearance in 4 experiments in normal dogs. The average inulin/creatinine clearance ratios for 3 to 4 consecutive clearance periods were 0.97, 1.00, 1.02, and 1.03.

The application of the anthrone reaction to other carbohydrates can be considerably extended, especially when controlled heating is possible. We have measured sucrose at 75°C, though at this temperature the standard curve is not as reproducible as it is for inulin. Simultaneous measurements of sucrose and inulin are possible since sucrose is yeast-labile. Applications of the anthrone method to the measurement of glycogen(10), glucose(11), dextran(12), and polyglucose(13) in biological materials have been reported. The presence of any of these materials except glucose would interfere with the inulin determination.

In our preliminary experiments using less anthrone (0.2%) we noted that digestion of high concentrations of glucose yields a product that diminishes inulin color development. This interference has been resolved by the use of higher concentrations of anthrone (0.4%).

The incomplete recovery of inulin from plasma apparent not only in our data, but also in other reports(14), probably represents loss during the precipitation of plasma protein. This loss is diminished by prolonged shaking of the precipitate. We have observed that sucrose recovery is higher than inulin recovery, suggesting that substances of higher molecular weight are more liable to be lost in plasma precipitates.

Summary. 1. A method for the determination of inulin in biological fluids using a modified anthrone reagent is described. 2. This reagent gives a sensitive and reproducible test for inulin in amounts of 15 to 45 μg. 3. Interfering substances are effectively removed from plasma filtrates and diluted urine by sodium hydroxide digestion. Ten to 25% of commercial inulin is found to be alkali-labile. 4. Recoveries from plasma and urine average 97.7 ± 2.5% and 99.7 ± 2.0%, respectively.

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Received July 22, 1952. P.S.E.B.M., 1952, v80.

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Prepared by Dr. William Antopol
(assisted by Dr. Howard Quittner)

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